

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization International Bureau



(43) International Publication Date  
17 June 2004 (17.06.2004)

PCT

(10) International Publication Number  
WO 2004/050859 A3

(51) International Patent Classification<sup>7</sup>: C12N 15/85, A61K 48/00

(74) Agent: LAWRENCE, William, E., Frommer Lawrence & Haug LLP, 745 Fifth Avenue, New York, NY 10151 (US).

(21) International Application Number: PCT/US2003/038811

(22) International Filing Date: 25 November 2003 (25.11.2003)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data: 60/429,631 27 November 2002 (27.11.2002) US

(71) Applicant (for all designated States except US): REGENTS OF THE UNIVERSITY OF MINNESOTA [US/US]; 110 Washington Avenue South, Minneapolis, MN 55455 (US).

(72) Inventors; and

(75) Inventors/Applicants (for US only): VERFAILLIE, Catherine [US/US]; 585 Cretin Avenue South, St. Paul, MN 55116 (US). LAKSHMIPATHY, Uma [US/US]; Stem Cell Institute, University of Minnesota, 420 Delaware Street SE, Minneapolis, MN 55455 (US).

(81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States (regional): ARIPO patent (BW, GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— with international search report

(88) Date of publication of the international search report: 12 August 2004

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 2004/050859 A3

(54) Title: HOMOLOGOUS RECOMBINATION IN MULTIPOTENT ADULT PROGENITOR CELLS

(57) Abstract: The invention relates to methods of altering gene expression by homologous recombination in a multipotent adult progenitor cell (MAPC). In particular, methods of producing a recombinant MAPC, of correcting a genetic defect in a mammal, of providing a functional and/or therapeutic protein to a mammal, and of transforming a MAPC are provided. MAPCs containing an exogenous DNA as well as recombinant MAPCs and their differentiated progeny are also provided.

TITLE OF THE INVENTIONHOMOLOGOUS RECOMBINATION IN MULTIPOTENT ADULT  
PROGENITOR CELLSRELATED APPLICATIONS

5 This application claims the benefit of priority to U.S. application Serial No. 60/429,631, filed on November 27, 2002. This application makes reference to International Application No. PCT/US00/21387, filed August 4, 2000 and to International Application No. PCT/US02/04652, filed February 14, 2002, both of which are hereby incorporated by reference. Each document cited or referenced in  
10 each of the foregoing applications, and any manufacturer's instructions or catalogues for any products cited or mentioned in each of the foregoing applications and in any of the cited documents, are hereby incorporated herein by reference. Furthermore, all documents cited in this text, and all documents cited or referenced in documents cited in this text, and any manufacturer's instructions or catalogues for any products cited or mentioned in this text or in any document incorporated into  
15 this text, are incorporated herein by reference. Documents incorporated by reference into this text or any teachings therein can be used in the practice of this invention. Documents incorporated by reference into this text are not admitted to be prior art.

FIELD OF THE INVENTION

20 The present invention relates generally to alteration of gene expression in mammalian multipotent adult progenitor cells (MAPCs) [previously referred to as multipotent adult stem cells (MASC)], and more specifically to methods for making homologously recombined MAPCs. The invention further relates to the therapeutic treatment of disease using homologous recombination and gene targeting techniques  
25 in MAPCs.

BACKGROUND OF THE INVENTION

The alteration of gene expression, by upregulating, downregulating, knocking-in or knocking-out gene products, can be accomplished using gene targeting approaches, such as homologous recombination. Gene targeting approaches are an alternative for both *in vitro* and *in vivo* production of proteins. *In vitro* expression of desired proteins has multiple uses, from production of therapeutic drugs to generating nutrients to providing drug and disease screening and

research tools. An aspect of gene targeting involves gene therapy, which has been advanced as a treatment for medical conditions that require alteration of the level of protein production in a cell, whether *ex vivo* or *in vivo*, and, if necessary, the delivery of such protein(s) to other cells and tissues.

5 An aspect of gene targeting *ex vivo* involves transfection and transplantation of recombinant autologous or allogeneic cells. As one example, stem cells provide promise for improving the results of such cell-based gene targeting. Stem cells can be genetically altered *in vitro*, then reintroduced *in vivo* to produce a desired gene product.

10 The quintessential stem cell is the embryonic stem (ES) cell, as it has unlimited self-renewal and multipotent differentiation potential (Thomson *et al.*, 1995; Thomson *et al.*, 1998; Shambrook *et al.*, 1998; Williams *et al.*, 1988; Orkin, 1998; Reubinoff *et al.*, 2000). These cells are derived from the inner cell mass of the blastocyst (Thomson *et al.*, 1995; Thomson *et al.*, 1998; Martin, 1981), or can be 15 derived from the primordial germ cells from a post-implantation embryo (embryonal germ cells or EG cells). ES and EG cells have been derived from mouse, and more recently also from non-human primates and humans. When introduced into mouse blastocysts, ES cells have been shown to contribute to tissues from all three germ layers of the mouse (Orkin 1998). ES cells are therefore pluripotent.

20 Stem cells that are not isolated from an embryo are simply referred to as stem cells or sometimes tissue-specific stem cells or adult stem cells. Stem cells have been identified in most organs and tissues. A well-characterized stem cell is the hematopoietic stem cell ("HSC"). This mesoderm-derived cell has been purified based on cell surface markers and functional characteristics. The HSC, isolated 25 from bone marrow ("BM"), blood, cord blood, fetal liver and yolk sac, is the progenitor cell that generates blood cells, or following translation, reinitiates multiple hematopoietic lineages. HSCs can reinitiate hematopoiesis for the life of a recipient. (See Fei *et al.*, U.S. Patent No. 5,635,387; McGlave *et al.*, U.S. Patent No. 5,460,964; Simmons *et al.*, U.S. Patent No. 5,677,136; Tsukamoto *et al.*, U.S. Patent No. 5,750,397; Schwartz *et al.*, U.S. Patent No. 5,759,793; DiGuistio *et al.*, U.S. Patent No. 5,681,599; Tsukamoto *et al.*, U.S. Patent No. 5,716,827; Hill *et al.*, 1996.) Stem cells which differentiate only to form cells of the hematopoietic lineage, however, are unable to provide a source of cells for repair of other damaged

tissues, for example, heart or lung tissue damaged by high-dose chemotherapeutic agents. They are also limited in their use in cell-based therapy to the correction of defects that affect only cells of the hematopoietic lineage. Similarly, their use in *in vitro* and/or *ex vivo* protein production is limited to proteins normally expressed in 5 cells of hematopoietic lineage.

A second adult stem cell that has been studied extensively is the neural stem cell ("NSC") (Gage, 2000; Svendsen *et al.*, 1999; Okabe *et al.*, 1996). Several studies in rodents, and non-human primates and humans have shown that stem cells continue to be present in adult brain. These stem cells can proliferate *in vivo* and 10 continuously regenerate at least some neuronal cells *in vivo*. When cultured *ex vivo*, NSCs can be induced to differentiate into different types of neurons and glial cells. Clarke *et al.* (2000) reported that NSCs from Lac-Z transgenic mice were able to contribute, not only to tissues of the central nervous system, but also to mesodermal derivatives and epithelial cells of the liver and intestine. They were not found in 15 other tissues, including the hematopoietic system. These studies therefore suggested that adult NSCs may have significantly greater differentiation potential than previously realized, but still do not have the pluripotent capability of ES cells or of the adult derived multipotent adult stem cells (MASCs), also known as MAPCs, described in Furcht *et al.* (International Application Nos. PCT/US00/21387 and 20 PCT/US02/04652) and herein.

A third tissue specific cell with stem cell properties is the mesenchymal stem cell ("MSC"), initially described by Fridenshtein (1982). MSC, originally derived from the embryonal mesoderm, can be isolated from adult bone marrow ("BM") and can differentiate to form muscle, bone, cartilage, fat, marrow stroma, and tendon. A 25 number of MSCs have been isolated. (See, for example, Caplan *et al.*, U.S. Patent No. 5,486,359; Young *et al.*, U.S. Patent No. 5,827,735; Caplan *et al.*, U.S. Patent No. 5,811,094; Bruder *et al.*, U.S. Patent No. 5,736,396; Caplan *et al.*, U.S. Patent No. 5,837,539; Masinovsky, U.S. Patent No. 5,837,670; Pittenger, U.S. Patent No. 5,827,740; Jaiswal *et al.*, 1997; Cassiede *et al.*, 1996; Johnstone *et al.*, 1998; Yoo *et al.*, 1998; Gronthos, 1994).

Of the many MSCs that have been described, most have demonstrated limited differentiation potential, only forming cells generally considered to be of mesenchymal origin. One of the most multipotent MSC reported is the cell isolated

by Pittenger *et al.*, which is capable of differentiating to form a number of cell types of mesenchymal origin (Pittenger *et al.*, 1999).

Other tissue-specific stem cells have been identified, including 5 gastrointestinal stem cells (Potten 1998), epidermal stem cells (Watt, 1997), and hepatic stem cells, also termed oval cells (Alison *et al.*, 1998).

MAPCs are distinct from these other types of stem cells. They can be culture-isolated from multiple sources, such as bone marrow, blood, muscle, brain, skin, fat, umbilical cord and placenta, and have the same morphology, phenotype, *in vitro* differentiation ability and a highly similar expressed gene profile as ES cells. 10 (See, for example, Reyes and Verfaillie, 2001; Reyes *et al.*, 2001; Jiang *et al.*, 2002a; Jiang *et al.*, 2002b.) MAPCs constitutively expresses oct4 and high levels of telomerase and are negative for CD44, MHC class I and MHC class II expression. One benefit of MAPCs, in terms of therapeutic applications, is that no teratomas are formed *in vivo*. Furthermore, MAPCs contribute to multiple organs upon 15 transplantation.

Most presently available approaches of gene delivery make use of infectious vectors, such as retroviral vectors, which include the genetic material to be expressed. These approaches have limitations, such as the potential of generating replication-competent virus during vector production; recombination between the 20 therapeutic virus and endogenous retroviral genomes, potentially generating infectious agents with novel cell specificities, host ranges, or increased virulence and cytotoxicity; limited cloning capacity in the retrovirus (which, *inter alia*, restricts therapeutic applicability) and short-lived *in vivo* expression of the product of interest.

Further, in most gene delivery systems, it is not possible to direct or target 25 the donor DNA (*i.e.*, the DNA being delivered to the cell, such as therapeutic DNA) to a preselected site in the genome. In fact, in the widely used retrovirus-mediated gene delivery system, retroviruses integrate randomly into independent chromosomal sites in millions to billions of cells. This mixture of infected cells is 30 problematic in two senses: first, since integration site plays a role in the function of the donor DNA, each cell has a different level of function and, second, since the integration of donor DNA into the genome can trigger undesired events, such as the

generation of tumorigenic cells, the likelihood of such events is dramatically increased when millions to billions of independent integrations occur.

The problems of populations consisting of large numbers of independent integrants might be avoided in two ways. First, a single cell with a random 5 integration site can be propagated until sufficient numbers of the cloned cell are available for further use. The cells that make up this clonal population would all function identically. While this is theoretically possible, success rates for creating a clonal population from a single cell can be low and the number of passages required to amass a usable number of transfected cells can be deleterious. Alternatively, gene 10 targeting can be used, wherein the donor DNA is introduced into a population of cells such that the DNA sequence integrates into a preselected site in the genome. In this case, all the cells function identically and the risk of a deleterious integration event is eliminated.

A number of approaches to gene targeting have been described including 15 chimeroplasty (Bandyopadhyay *et al.*, 1999), triple helix formation (Casey *et al.*, 2001) and short-fragment homologous recombination (Goncz, *et al.*, 2001), all of which may increase the rate of gene targeting. A preferred method of gene targeting by homologous recombination is that of Treco *et al.*, described, for example, in the USPN 6,270,989 and 5,641,670 or in Selden *et al.*, USPN 6,303,379.

20 Another alternative approach, based on AAV-mediated gene transfer and targeting, has been described (Inoue *et al.*, 1999; Hirata *et al.*, 2000; Hirata *et al.*, 2002). AAV is a dependent parvovirus with a single-stranded linear DNA genome, from which vectors can be made by replacing the viral genes with foreign DNA between the cis-acting inverted terminal repeats. AAV vectors genetically alter cells 25 by chromosomal integration of the vector genome at the site-specific integration locus of wild-type AAV located on human chromosome 19 (Carter *et al.*, 2000; Inoue *et al.*, 1999; Hirata *et al.*, 2000; Hirata *et al.*, 2002). The gene targeting rates produced by AAV vectors approach 1% at the single-copy HPRT locus in normal human cells, 3 to 4 logs higher than can typically be achieved in human cells with 30 conventional gene targeting methods.

The use of gene targeting has been proposed in ES cell, germ cell and somatic cell systems. Germ cell gene targeting refers to the modification of sperm cells, egg cells, zygotes, or early stage embryos. From a practical standpoint, and

due to ethical concerns, germ cell gene targeting is inappropriate for human use. ES cell gene targeting is also controversial, and the availability of ES cells for these purposes is severely limited. In somatic cell gene therapy, targeting somatic cells (e.g., fibroblasts, hepatocytes, or endothelial cells) are removed from a donor 5 organism, cultured *in vitro*, transfected with the gene(s) of interest, characterized, and used for a desired purpose. However, the practical use of somatic cells is limited to conditions that affect only one cell type. Therefore, for example, an altered somatic cell cannot be induced to differentiate into cells of various tissue types. In addition, somatic cells are generally limited in their potential to propagate 10 *in vitro*. Hence, an approach that overcomes the drawbacks and limitations of the currently available methods and provides safe, efficacious, long-term protein production and delivery would be valuable.

Historically, transfer (or introduction) of exogenous DNA into stem cells has been challenging, with most of the known transfection methods giving sub-optimal 15 transfer rates. To achieve efficient gene targeting, a good transfer rate is necessary. Achieving high rates of transfer in stem cells has been hindered by the fact that optimal transfection occurs when cells are cultured at high density, while lower cell densities are required to maintain stem cells in an undifferentiated state. In order to effectively use genetically altered stem cells in protein expression, including 20 therapeutic applications, a method is needed that will result in optimal transfer efficiency, for example, under conditions that support undifferentiated stem cells. Moreover, such methods would be ideally suited for use with adult stem cells having 25 pluripotent capacity, such as MAPCs.

#### SUMMARY OF THE INVENTION

25 The ability to repair, alter, replace, delete or express desired nucleotide sequences in the genome of MAPCs would expand the potential usefulness of MAPCs in the *in vitro*, *ex vivo*, or *in vivo* expression of a gene of interest in order to arrive at a multitude of desired outcomes, such as nutritional and/or therapeutic protein production, non-protein gene expression (e.g. generation, up/down 30 regulation, or knock-out/-in of ribozymes), as well as treatment of disease. Use of gene targeting in MAPCs can provide methods for altering gene expression (i.e., by increasing or decreasing the production of a gene product) not only to produce a

novel or enhanced gene product, but also for investigation of gene expression patterns and/or gene function.

The invention provides a method of altering a first gene expression pattern in an isolated multipotent adult progenitor cell (MAPC), the method comprising:

- 5        a) introducing into a MAPC an exogenous polynucleotide molecule, wherein the polynucleotide molecule comprises i) a targeting nucleotide sequence and ii) a donor nucleotide sequence; and
- b) culturing the MAPC under conditions sufficient to homologously recombine the exogenous polynucleotide molecule, such that a resultant

10        MAPC has a second expression pattern;

      wherein the introducing and culturing are done simultaneously or consecutively.

A preferred polynucleotide molecule is a DNA molecule. A preferred targeting and/or donor nucleotide sequence is DNA. Preferably, a targeting nucleotide sequence is homologous to a genomic DNA sequence of the MAPC.

- 15        More preferably, a donor nucleotide sequence encodes a gene product not expressed by the MAPC, such that the second expression pattern includes the gene product.

Gene expression in the resultant MAPC can be turned on or off, increased or decreased.

MAPCs can be isolated from a mammal, such as a mouse, a rat or a human.

- 20        MAPCs can be isolated from a source including, but not limited to, bone marrow, blood, brain, muscle, skin, fat, umbilical cord and placenta.

The exogenous DNA sequence can be introduced via nucleoporation.

- The method of altering a first gene expression pattern in an isolated multipotent adult progenitor cell (MAPC) can further comprise differentiating the
- 25        MAPC after culturing under conditions sufficient to homologously recombine the exogenous polynucleotide molecule (preferably a DNA molecule). The MAPC can be differentiated to form a cell of a type including, but not limited to, osteoblast, chondrocyte, adipocyte, fibroblast, marrow stroma, skeletal muscle, smooth muscle, cardiac muscle, ocular, endothelial, epithelial, hepatic, pancreatic, hematopoietic, glial, neuronal and oligodendrocyte cell types.

The preferred exogenous DNA molecule can comprise a DNA sequence encoding a selectable marker, such as an antibiotic resistance gene.

The invention further provides a method of making recombinant multipotent adult progenitor cells (MAPCs), the method comprising:

- a) culturing isolated MAPCs at low density;
- b) nucleoporating the MAPC in the presence of an exogenous DNA molecule, wherein the DNA molecule comprises i) a DNA sequence homologous to a genomic DNA sequence of the MAPC and ii) optionally a DNA sequence encoding a gene product; and
- c) culturing the MAPC under conditions sufficient to homologously recombine the exogenous DNA molecule.

10 Preferably, the MAPCs are cultured at about 500 cells/cm<sup>2</sup>.

The gene product can be, but is not limited to, FANCA, FANCB, FANCC, FANCD1, FANCD2, FANCE, FANCF, FANCG, hemoglobin, factor VIII, factor IX, erythropoietin, CFTR, collagen I, alpha 1 anti-trypsin, gamma-C, Rag1, Rag2, adenosine deaminase, phenylalanine hydroxylase, fumarate dehydroxylase, LDL receptor, aL-iduronidase and b-glucuronidase. Preferably, the gene product is FANCC.

The method of making recombinant multipotent adult progenitor cells can further comprise selecting the resultant MAPCs expressing the gene product.

15 The preferred exogenous DNA molecule can be a vector, such as a plasmid. The vector can further comprise a DNA sequence encoding a selectable marker, such as an antibiotic resistance gene.

The invention further provides a method of correcting a genetic defect in a mammal, wherein the defect is one or more defective nucleotide sequence(s) from which a functional gene product cannot be expressed, the method comprising:

- a) isolating a MAPC from the mammal having the genetic defect;
- b) introducing into the MAPC an exogenous DNA molecule, wherein the DNA molecule comprises i) a DNA sequence homologous to a genomic DNA sequence of the MAPC and ii) one or more non-defective nucleotide sequence(s) corresponding to the defective nucleotide sequence(s),
- c) culturing the MAPC under conditions sufficient to homologously recombine the exogenous DNA molecule into the genome of the MAPC, wherein the MAPC expresses the functional gene product;

- d) selecting MAPCs that express the functional gene product; and
- e) transplanting the MAPCs into the mammal.

The genetic defect can affect a gene encoding, for example, FANCA, FANCB, FANCC, FANCD1, FANCD2, FANCE, FANCF, FANCG, hemoglobin, factor VIII, factor IX, erythropoietin, CFTR, collagen I, alpha 1 anti-trypsin, gamma-C, Rag1, Rag2, adenosine deaminase, phenylalanine hydroxylase, fumarate dehydroxylase, LDL receptor, aL-iduronidase and b-glucuronidase.

Selection can be carried out by treatment of the MAPCs with a dose of mitomycin C, wherein the dose is toxic to MAPCs not expressing the gene product and non-toxic to MAPCs expressing the gene product.

The method of correcting a genetic defect can further comprise differentiating the MAPCs. The differentiating can be done after culturing under conditions sufficient to homologously recombine the exogenous DNA molecule and optionally, before transplanting the MAPCs into the mammal.

The exogenous DNA molecule can further comprise a DNA sequence encoding a selectable marker, such as an antibiotic resistance gene. Thus, the method of correcting a genetic defect can further comprise selecting MAPCs expressing the selectable marker after culturing under conditions sufficient to homologously recombine the exogenous DNA molecule.

Preferably, the sequence encoding the selectable marker can be excised from the genome of a resultant MAPC. Most preferably, the sequence encoding the selectable marker is flanked at each of the 5' and 3' ends by a *lox* P site. Thus, the method of correcting a genetic defect can further comprise selecting MAPCs expressing the selectable marker after culture under conditions sufficient to homologously recombine the exogenous DNA molecule and introducing into the

MAPCs a vector comprising a gene encoding Cre, such that Cre is expressed and the selectable marker is excised. In a preferred embodiment, the selectable marker is excised before transplanting the MAPCs into the mammal.

The vector comprising a gene encoding Cre can be an adeno-associated viral vector.

The method of correcting a genetic defect can further comprise selecting MAPCs expressing the selectable marker after culturing under conditions sufficient to homologously recombine the exogenous DNA molecule and introducing into the

MAPCs a TaT-Cre fusion protein, such that the selectable marker is excised. In a preferred embodiment, the selectable marker is excised before transplanting the MAPCs into the mammal.

5 The invention further provides a MAPC, or the differentiated progeny thereof, isolated from a mammal with a genetic defect, wherein the defect is one or more defective nucleotide sequence(s) at a known locus such that a functional gene product is not expressed, and wherein the MAPC comprises a DNA molecule with one or more non-defective nucleotide sequence(s) corresponding to the known locus, and the MAPC expresses the functional gene product.

10 The invention further provides a method of expressing a functional gene product in an isolated MAPC having a defective nucleotide sequence from which a functional gene product cannot be expressed, the method comprising:

- 15 a) introducing into the MAPC an exogenous DNA molecule, wherein the DNA molecule comprises i) a DNA sequence homologous to a genomic DNA sequence of the MAPC and ii) a non-defective nucleotide sequence corresponding to the defective nucleotide sequence; and
- b) culturing the MAPC under conditions sufficient to homologously recombine the exogenous DNA molecule into the genome of the MAPC, wherein the MAPC expresses the functional gene product.

20 In a preferred embodiment, the defective nucleotide sequence is in FANCC.

The method of expressing a functional gene product in an isolated MAPC can further comprise selecting the MAPCs that express the functional gene product.

25 The invention further comprises a recombinant MAPC produced by the methods of the invention.

The term "comprising" in this disclosure can mean "including" or can have the meaning commonly given to the term "comprising" in U.S. Patent Law.

Other aspects of the invention are described in or are obvious from (and within the ambit of the invention) the following disclosure.

### 30 **BRIEF DESCRIPTION OF DRAWINGS**

The following Detailed Description, given by way of example, but not intended to limit the invention to specific embodiments described, may be

understood in conjunction with the accompanying drawings, incorporated herein by reference, in which:

Figure 1 shows the percentage of cells expressing GFP after transfection using various methods.

Figure 2 shows the percentage of cells expressing GFP at 24 and 48 hours after transfection by electroporation (Ep) or nucleoporation (Np).

Figure 3 shows the HPRT gene and the vector used to target it in MAPCs.

Figure 4 shows the gene targeting frequency in mMPCs compared with the frequency in HSC and ES cells (Hatada *et al.*, 2001).

Figure 5 provides an overview of the experimental strategy for gene repair in a knockout mouse model system.

Figure 6 shows FANCC-/- MAPCs that have differentiated into cardiac cells (6A), liver cells (6B), endothelial cells (6C), muscle cells (6D) and neurons (6E).

Figure 7 shows schematic diagrams of the FANCC gene, containing 14 exons, the knockout locus in Fanconi anemia model mice, and the correction vector used to correct the defective FANCC gene.

Figure 8 shows the nucleotide sequence of the human FANCC cDNA (SEQ ID NO:1).

Figure 9 shows the amino acid sequence of the human FANCC protein (SEQ ID NO:2).

#### **DETAILED DESCRIPTION OF THE INVENTION**

As described herein, MAPC technology, in combination with homologous recombination, provides a powerful tool for altering gene expression to produce a gene product *in vitro*, to determine the function of a gene and to treat diseases and conditions caused by abnormal gene function. The methods and cells of the present invention can be used for a wide variety of purposes. The methods can be used to alter MAPCs in order to repair, alter, delete or replace DNA already present in the MAPC, to activate/knock in a gene or to add or replace regulatory sequences to a MAPC, to knock out or remove an entire gene or gene portion present in MAPCs, or to introduce a gene or DNA sequence into a MAPC, at a preselected site, which encodes a therapeutic product or other desired product.

Accordingly, the invention provides a method of altering a first gene expression pattern in an isolated multipotent adult progenitor cell (MAPC), the method comprising:

- (a) introducing into the MAPC an exogenous polynucleotide molecule, wherein the polynucleotide molecule comprises i) a targeting nucleotide sequence which includes a sequence homologous to a genomic nucleotide sequence of the MAPC and ii) a donor nucleotide sequence; and
- (b) culturing the MAPC under conditions sufficient to homologously recombine the exogenous polynucleotide molecule, such that a resultant MAPC has a second gene expression pattern different than said first gene expression pattern.

MAPCs can be isolated from a mammal such as a mouse, a rat or a human.

MAPCs can be isolated from a source including, but not limited to, bone marrow, blood, brain, muscle, skin, fat, umbilical cord and placenta.

As used herein, the term "isolated" as used in "isolated MAPC," or "isolated MAPCs" refers to one or more MAPCs harvested from a source. A preferred method of harvesting a MAPC is described in PCT/US00/21387 (published as WO 01/11011) and PCT/US02/04652 (published as WO 02/064748); MAPC compositions and the methods for the isolation thereof are incorporated herein by reference.

In general, "homologous recombination" is the substitution of a segment of DNA by another that is identical or nearly so. As used herein, to homologously recombine an exogenous DNA molecule is to physically exchange segments of the molecule, preferably segments flanking the donor nucleotide sequence, with homologous genomic DNA sequences of the MAPC, such that the exogenous DNA molecule, or a portion thereof, is inserted into the MAPC genome. For a review of homologous recombination, see Lewin, B., *Genes V*, Oxford University Press, New York, 1994, pp. 968-997; and Capecchi, M., (1989) *Science* 244:1288-1292; 30 Capecchi, M., (1989) *Trends Genet.* 5(3):70-76.

As used herein, an "exogenous polynucleotide molecule," can comprise a polynucleotide sequence obtained from a source other than the very cell into which

it is introduced. The polynucleotide can comprise a sequence of synthetic or naturally occurring DNA or RNA nucleotide bases.

As used herein, a "targeting nucleotide sequence," is a sequence that shares homology with a genomic sequence(s) of the MAPC. For example, where gene activation is desired (e.g., when the donor sequence comprises a transcriptional regulatory sequence, such as a transcriptional activation or enhancer sequence), the targeting nucleotide sequence can have homology with a genomic sequence within the gene to be activated or upstream of the gene to be activated, the upstream region being up to and including the first functional splice acceptor site on the same coding strand of the gene of interest, and by means of which homology the donor sequence that activates the gene of interest is integrated into the genome of the cell containing the gene to be activated. Where insertion of a donor sequence into a coding region of a gene is desired, the targeting nucleotide sequence can share homology with a genomic sequence, preferably, in an exon of that gene. Where insertion of a donor sequence into a non-coding region is desired (e.g., insertion of a donor sequence without interfering with transcription of endogenous genes is desired), the targeting nucleotide sequence can share homology with a genomic sequence that is, preferably, not located within a transcribed genomic locus. A "genomic locus" refers to a particular location within the genome (e.g., the location of a gene).

As used herein, a "donor nucleotide sequence" is any nucleotide sequence that is intended to be inserted into the MAPC genome regardless of its size. The donor nucleotide sequence can comprise, for example, a coding sequence, or a portion thereof, a transcriptional regulatory sequence, such as a transcriptional activation, enhancer or silencer sequence, or a non-coding sequence, such as a knock-out cassette, as well as a single nucleotide.

In general, in the various methodologies of the present invention, preferably, the polynucleotide molecule is DNA; and the exogenous polynucleotide molecule is contained in a vector. Moreover, the targeting and the donor nucleotide sequence of the exogenous polynucleotide molecule may be one and the same.

Introduction into the MAPC of an exogenous polynucleotide molecule, preferably a DNA molecule, can be through any methodology, such as transfection, liposome fusion or electroporation. Preferably, introduction into the MAPC of the exogenous polynucleotide molecule is carried out through nucleoporation.

Gene expression in the resultant MAPC can be turned on, increased, turned off or decreased. The methods of the invention can result in addition, deletion or replacement of all or part of a gene in a MAPC. Such manipulation of the genome is useful in several respects. The method is particularly useful to turn on or increase expression of a gene that is present in a MAPC, but is not expressed at all or in biologically significant levels (i.e. such that a functional gene product is produced). Once gene expression is altered, for example, turned on or increased, the resultant MAPC can be used to produce the gene product (i.e., protein or RNA molecule) *in vitro* or *in vivo*. The suppression of gene expression can be useful in determining the function of a gene product, or in gene mapping as well as correcting a genetic defect. In addition, when a gene product is not expressed, or is expressed in a reduced amount, the relative overexpression of other gene products can be simulated. Such techniques can be useful, for example, in the generation of disease models, such as cancer.

The resultant MAPC, having the second gene expression pattern, can be selected on that basis. Accordingly, the invention further provides a method of identifying a homologously recombined multipotent adult progenitor cell (MAPC) by selecting for an effect of the donor nucleotide on said MAPC. Thus, recombinant MAPCs produced by the above methods are also provided by the invention.

As used herein, a "recombinant MAPC," is a MAPC comprising a donor nucleotide sequence inserted into its genome. As used herein, a "genetically altered MAPC" is a MAPC whose genome is altered, as a result of homologous recombination, by at least one nucleotide deletion, substitution, addition. "Substitution" can include the exchange of a genomic nucleotide base(s) for either the same or a different nucleotide base(s) of an exogenous polynucleotide molecule (e.g., a genomic guanine exchanged for an exogenous guanine or a genomic guanine exchanged for an exogenous thymine).

In a preferred embodiment, the invention provides a method of making a recombinant multipotent adult progenitor cell (MAPC), comprising:

- 30 (a) culturing MAPCs;
- (b) nucleoporating the MAPC in the presence of an exogenous DNA molecule, wherein the DNA molecule comprises i) a DNA sequence

homologous to a genomic DNA sequence of the MAPC and ii) optionally a DNA sequence encoding a gene product; and

5 (c) culturing the MAPC obtained in (b) under conditions sufficient to homologously recombine the exogenous DNA molecule, thereby making a recombinant MAPC.

In a preferred embodiment of the above method, the MAPCs of (a) and/or (c) are cultured at low density, preferably at about 500 cells/cm<sup>2</sup>.

In another embodiment, the invention provides a method of introducing a nucleotide of interest ("NOI") into multipotent adult progenitor cells (MAPCs) comprising:

(a) culturing MAPCs at low density, preferably at about 500 cells/cm<sup>2</sup>;

(b) introducing a vector comprising a nucleotide of interest ("NOI") into the MAPCs using nucleoporation.

Frequently, a genetic disorder or disease results from error(s) in the genetic code in an organism's genome, wherein a mutation of one or more nucleotides causes alteration(s) in the gene expression, such as over or under expression, activation or deactivation of a gene. As used herein "genetic code" refers to a sequence of nucleotides, coded in triplets ("codons") along an RNA sequence.

Errors in the genetic code can occur in one or more gene loci, within or outside a coding and/or regulatory region. A single nucleotide mutation can alone or cumulatively (and/or synergistically) with one or more single nucleotide mutation give rise to a disease or disorder in the whole cell, tissue and/or organism. For example, mutations in genes affecting a protein function such as anemia, diabetes, hepatitis, hemophilia, hemoglobinopathies, muscular dystrophy and cystic fibrosis, to name a few, could be corrected by methods of the invention.

For such conditions, the invention further provides a method of correcting a genetic defect in a mammal, wherein the defect is one or more defective nucleotide sequence(s) in the genome of the mammal that give rise to defective gene expression, the method comprising:

30 (a) culturing a MAPC from the mammal having the genetic defect;

(b) introducing into the MAPC an exogenous DNA molecule, wherein the DNA molecule comprises i) a targeting DNA sequence homologous to a genomic DNA sequence of the MAPC and ii) one or

more donor nucleotide sequence(s) necessary for correcting said genetic defect in said mammal;

- (c) culturing the MAPC under conditions sufficient to homologously recombine the exogenous DNA molecule into the genome of the MAPC, thereby obtaining a genetically altered MAPC;
- (d) selecting said genetically altered MAPC; and
- (e) transplanting said genetically altered MAPC into the mammal, wherein the selecting recited in (d) and the transplanting recited in (e) can be done in any order or simultaneously.

10 Preferably, the exogenous DNA molecule is contained in a "correction vector." As used herein, a "correction vector" is comprised of a replicon (i.e., a polynucleotide molecule capable of independently replicating, such as a plasmid or viral backbone), that contains an exogenous polynucleotide molecule having i) a targeting nucleotide sequence and ii) one or more donor nucleotide sequence(s).

15 Prior to introduction into a MAPC of interest, the correction vector can be linearized at a site, preferably, outside the targeting nucleotide sequence or the donor nucleotide sequence. Preferably, the sequence homology between the genomic nucleotide sequence of the MAPC and the targeting nucleotide sequence includes either a region from within or proximal to the genomic locus of the genetic defect.

20 Another preferred embodiment involves a method of expressing a functional gene product in an isolated MAPC having a defective genetic code from which a functional gene product cannot be expressed, said method comprising:

- (a) introducing into the MAPC an exogenous DNA molecule, wherein the DNA molecule comprises i) a targeting DNA sequence homologous to a genomic DNA sequence of the MAPC and ii) a donor nucleotide sequence corresponding to the defective genetic code; and
- (b) culturing the MAPC under conditions sufficient to homologously recombine the exogenous DNA molecule into the genome of the MAPC, wherein the MAPC expresses the functional gene product.

30 In addition to the instances when undifferentiated recombinant MAPCs are administered to a patient and then differentiated into specific cells *in vivo*, the progeny of the recombinant MAPCs can be differentiated *ex vivo* before, after, or

concurrent with selection and then be administered to provide a desired, e.g., a therapeutic, benefit. The differentiated cells may be administered as a mixture of non-homogenous cells (i.e., cells at various stages of differentiation and/or in admixture with one or more different kind(s) of cells) or as cells of various degrees of purity/homogeneity in type and stage of development. The cells can be differentiated to form osteoblasts, chondrocytes, adipocytes, fibroblasts, marrow stroma, skeletal muscle cells, smooth muscle cells, cardiac muscle cells, ocular cells, endothelial cells, epithelial cells, hepatic cells, pancreatic cells, hematopoietic cells, glial cells, neuronal cells, oligodendrocytes, or any other type of specialized cell.

10 In a preferred embodiment, the MAPC is isolated from a mammal, such as a mouse, a rat, a human or other primate, as well as a dog or pig. Most preferably the MAPC is isolated from a human.

15 MAPCs can be isolated from most organ, tissue and fluid sources, including, but not limited to, BM, blood, brain, heart, muscle, skin, fat, umbilical cord and placenta. In a preferred embodiment, MAPCs are isolated from BM, brain, heart or muscle. In an especially preferred embodiment, MAPCs are isolated from BM, heart or muscle.

20 The exogenous DNA molecule can be introduced into the MAPC in a vector, or correction vector, which can be any vector comprising a polynucleotide sequence, e.g. a plasmid vector or a viral vector. In a preferred embodiment, it is a plasmid. The vector can further comprise a DNA sequence encoding a selectable marker, optionally flanked at the 5' and 3' ends by *lox P* sites. The selectable marker is preferably an antibiotic resistance gene. In this embodiment, the methods of the invention can further comprise the step of selecting cells expressing the selectable marker prior to selecting cells expressing the gene product or protein. The methods of the invention can also further comprise introducing the Cre enzyme into the cells prior to transplantation, such that the selectable marker is removed from the genome. Cre can be introduced in any manner, preferably, either by transfection of the cells with a vector comprising the *cre* gene, or by crosslinking the polypeptide to the HIV 25 trans-activating transduction (Tat) peptide and introducing it into the cells.

30 Selection of homologously recombined cells can be *in vitro*, prior to transplantation of the cells, and/or *in vivo*, e.g., after transplantation has occurred. *In vivo* selection can be by administration of a drug to which untransfected cells are

susceptible, such as mitomycin C, or it can be by means of determining expression of a functional gene product by methods known in the art. Phenotypic selection, for example by histological analysis or by the reduction of symptoms manifested by the genetic defect, may also be used as a criterion. The preferred dose of mitomycin C for *in vivo* selection is between about 0.3 mg/kg/week and 1 mg/kg/week.

The invention further provides a MAPC isolated from a mammal with a genetic defect, the defect arising from mutation of one or more nucleotide(s) on the mammal's genome. The mutation is, for example, responsible for inability of the cell to express a sequence(s) from which a functional gene product can be expressed.

10 The MAPC of the present invention comprises a genetically altered MAPC containing an exogenous polynucleotide molecule, such as a DNA molecule, wherein the polynucleotide molecule comprises i) a targeting polynucleotide sequence homologous to a genomic DNA sequence of the MAPC and ii) one or more donor nucleotide sequence(s) necessary for correcting the genetic defect. In a 15 preferred embodiment, the targeting and/or donor nucleotide sequences are DNA and correspond to the defective nucleotide sequence(s) at the gene locus responsible for the genetic defect, such that the exogenous DNA molecule is integrated into the genome of the cell and the cell expresses the functional gene product. A differentiated cell derived from this genetically altered MAPC is also provided by 20 the invention. The differentiated cell can be an osteoblast, chondrocyte, adipocyte, fibroblast, marrow stroma, skeletal muscle, smooth muscle, cardiac muscle, ocular, endothelial, epithelial, hepatic, pancreatic, hematopoietic, glial, neuronal, oligodendrocyte, or any other type of differentiated cell.

25 The present invention is useful for conducting targeted gene therapy in multipotent adult progenitor cells (MAPCs) from a mammal, preferably a human, other primates, mouse, rat, dog, and pig, to produce therapeutic gene products in a wide variety of cell and tissue types. A contemplated application of this technology is the method of correcting a genetic defect in a mammal by administering a therapeutically effective amount of the genetically altered MAPCs or their progeny.

30 The methods of the invention can be used to treat any disease or condition caused by a genetic deficiency in a known gene, including metabolic diseases, storage diseases, muscular dystrophy, osteogenesis imperfecta, severe combined immunodeficiency, liver disease, diabetes, hepatitis, hemophilia, hemoglobinopathies, anemia, and

cystic fibrosis. An unexpected benefit of this approach is that the need for pretreatment and/or post treatment of the patient with irradiation, chemotherapy, immunosuppressive agents or other drugs or treatments is reduced or eliminated. The induction of tolerance before or during treatment is also not required; however, 5 allogenic application is within the scope of the invention.

Another application of the invention is to provide a method for stably transfecting MAPCs under conditions that result in efficient transfection rates at cell densities that are sufficiently low to maintain MAPCs in an undifferentiated state.

MAPCs can be derived from a non-embryonic organ, tissue or fluid, and 10 have the capacity to differentiate to form cell types of mesodermal, ectodermal and endodermal origin. Differentiation can be induced *in vivo* or *ex vivo*, to produce a population of MAPC progeny. The progeny can have the capacity to be further differentiated, or can be terminally differentiated.

The invention, preferably, provides a method of altering a first gene 15 expression pattern in an isolated multipotent adult progenitor cell (MAPC), the method comprising:

- (a) introducing into the MAPC an exogenous DNA molecule, wherein the DNA molecule comprises i) a targeting DNA sequence homologous to a genomic DNA sequence of the MAPC and ii) a donor DNA sequence encoding a gene product not normally expressed by the MAPC; and
- (b) culturing the MAPC under conditions sufficient to homologously recombine the exogenous DNA molecule into the genome of said MAPC, such that a resultant MAPC has a second gene expression 25 pattern that includes the gene product.

The targeting DNA sequence and the donor DNA sequence can be one and the same or different.

Preferred methods of MAPC isolation are described in PCT/US00/21387 (published as WO 01/11011) and PCT/US02/04652 (published as WO 02/064748), 30 and these methods are incorporated herein by reference. MAPCs can be isolated from multiple sources, including bone marrow, muscle, brain, spinal cord, blood or skin. To isolate MAPCs, bone marrow mononuclear cells can be derived from bone marrow aspirates, which can be obtained by standard means known to those of skill

in the art (see, for example, Muschler, G.F., et al., *J. Bone Joint Surg. Am.* (1997) 79(11): 1699-709, Batinic, D., et al., *Bone Marrow Transplant.* (1990) 6(2): 103-7).

MAPCs are present within the bone marrow (or other organs, such as liver and brain), but do not express the common leukocyte antigen CD45 or erythroblast specific glycophorin-A (GlyA). The mixed population of cells can be subjected to a Ficoll Hypaque separation. Cells can then be subjected to negative selection using anti-CD45 and anti-Gly-A antibodies, depleting the population of CD45<sup>+</sup> and GlyA<sup>+</sup> cells, and recovering the remaining approximately 0.1% of marrow mononuclear cells. Cells can also be plated in fibronectin coated wells and cultured as described below for 2-4 weeks after which the cells are depleted of CD45<sup>+</sup> and GlyA<sup>+</sup> cells. Alternatively, positive selection can be employed to isolate cells using a combination of cell-specific markers, such as the leukemia inhibitory factor (LIF) receptor. Both positive and negative selection techniques are known to those of skill in the art, and numerous monoclonal and polyclonal antibodies suitable for negative selection purposes are also known in the art (see, for example, LeukocZe Typing V, Schlossman, et al., Eds. (1995) Oxford University Press) and are commercially available from a number of sources.

Preferable ranges of homogeneity in populations comprising MAPCs are 50-55%, 55-60%, and 65-70%. More preferably the homogeneity is 70-75%, 75-80%, 80-85%; and most preferably the homogeneity is 85-90%, 90-95%, and 95-100%.

Homogeneity of MAPCs can be determined according to the cell surface marker profile within a population.

Gene expression in the resultant MAPC of the methods of present invention is turned on, increased, turned off or decreased. By a "expression pattern" is meant the structure of and approximate amount of a gene product that is produced by a cell under specific conditions. Expression patterns can be determined by any methods known in the art, for example, by quantitative Western blot, amino acid sequence analysis, and/or protein concentration assays. "Normally expressed" refers to the structure of and approximate amount of a gene product that is produced by a cell, under specific conditions, that does not have a genetic defect in the nucleotide sequence encoding the gene product. "Gene product" as used herein and commonly understood in the art refers to a nucleic acid molecule (such as RNA) and/or a peptide or polypeptide encoded by the gene of interest.

“Conditions suitable for homologous recombination” include cell culture conditions whereby cells are plated at a density and in a medium that allows cells to be grown without differentiation during the recombination event. One example of suitable culture conditions would be 5x 10<sup>3</sup> MAPCs in 200  $\mu$ L medium comprising 5 58% DMEM-LG, 40% MCDB-201 (Sigma Chemical Co, St Louis, MO); supplemented with 1X insulin-transferrin-selenium (ITS), 1X linoleic-acid bovine serum albumin (LA-BSA), 10<sup>-8</sup> M Dexamethasone, 10<sup>-4</sup> M ascorbic acid 2-phosphate (all from Sigma), 100 U penicillin and 1,000 U streptomycin (Gibco) and 10-10% fetal calf serum (FCS) (Hyclone Laboratories, Logan, UT) with 10 ng/ml of EGF (Sigma) and 10 ng/ml PDGF-BB (R&D Systems, Minneapolis, MN). For detailed protocols, see Hatada et al. (2001) and Yanez and Porter ACG (1999).

Following homologous recombination, cells containing a donor nucleotide sequence can be selected in culture. The length of selection will vary, depending on the selectable marker, or combination of selectable markers, used. Selectable 15 marker genes which can be used include, but are not limited to neo, gpt, dhfr, ada, pac, hyg, mdrl, hisD, HSVTK and blastocidin. The selectable marker is preferably an antibiotic resistance gene. Most preferably, the hygromycin resistance (Hyg<sup>R</sup>) gene is used as a selectable marker gene, and cells containing Hyg<sup>R</sup> are cultured for at least 24 hours, up to about 7 days, most preferably for about 2 days to about 5 20 days, in the presence of hygromycin. Where more than one selectable marker gene is used, a second round of selection can be carried out using, for example, the selectable marker genes neomycin, HSVTK and blastocidin. Thus, after the initial time for selection (e.g., 24 hours to about 7 days) a second period of selection can be carried out according to the selectable marker gene of choice. For example, 25 selection for neomycin resistance can be performed for about 7 to about 14 days, while HSVTK and blastocidin resistance can be performed for about 5 to about 7 days. It is well within the skill in the art to vary the duration of selection for a particular selectable marker gene according to the time known in the art to be effective for selection.

30 Where the selectable marker gene is homologously recombined into the HPRT locus, endogenous HPRT is disrupted and cells are no longer sensitive to media containing 6-thioguanine (6-TG). One example of a dual selection method

which employs both 6-thioguanine (6-TG) and Hyg<sup>R</sup> selection is provided in Example 6. Other examples can be found in the art.

In a preferred embodiment, the invention provides a method of introducing a nucleotide of interest ("NOI") into multipotent adult progenitor cells (MAPCs) comprising the steps of:

- (a) culturing isolated MAPCs at low density, preferably at about 500 cells/cm<sup>2</sup>;
- (b) introducing a vector comprising a nucleotide of interest ("NOI") into the MAPCs using nucleoporation.

Introduction of the vector can be carried out by any method, including, but not limited to, liposome fusion, electroporation, or nucleoporation. Nucleoporation is the preferred mode of transfection, as this method is believed to allow high transfection efficiencies at low cell densities. Nucleoporation solves the paradox faced in the transfection of MAPCs: the rate of transfection must be high enough to produce a sufficiently large population of genetically altered cells to use in therapeutic applications, but the density of MAPCs in culture must be low enough to prevent differentiation and loss of multipotency.

A "nucleotide of interest" (NOI) can be any nucleic acid sequence, which need not necessarily be a complete naturally occurring DNA or RNA sequence. Thus, the NOI can be, for example, a synthetic RNA/DNA sequence; a recombinant RNA/DNA sequence (*i.e.* prepared by use of recombinant DNA techniques); a cDNA sequence or a partial genomic DNA sequence; including combinations thereof. The sequence need not be a coding region. If it is a coding region, it need not be an entire coding region. In addition, the RNA/DNA sequence can be in a sense orientation or in an anti-sense orientation. Preferably, it is in a sense orientation. Preferably, the sequence is, comprises, or is transcribed from cDNA. The NOI can be useful for assay development, protein production, therapeutic or diagnostic applications.

"Low density" refers to cell densities of about 0.5-1.5 x 10<sup>3</sup> cell/cm<sup>2</sup>, preferably about 1.0 x 10<sup>3</sup> cell/cm<sup>2</sup>, and even more preferably about 0.5 - 2.0 x 10<sup>3</sup> cell/cm<sup>2</sup>. most preferably, a "low density" refers to a cell density of 0.5 x 10<sup>3</sup> cell/cm<sup>2</sup>. Advantageously, the transfection efficiency is at least about 15%, preferably at least about 20%, and most preferably at least about 25% at 24 hours

post-transfection, and at least about 20%, preferably at least about 25%, more preferably at least about 30% and most preferably at least about 35% at 48 hours post-transfection.

5 In a preferred embodiment, the invention further provides a method of correcting a genetic defect in a mammal, wherein the defect is one or more defective nucleotide sequence(s) from which a functional gene product cannot be expressed, the method comprising:

- (a) isolating a MAPC from the mammal having the genetic defect;
- (b) introducing into the MAPC an exogenous DNA molecule, wherein the DNA molecule comprises i) a DNA sequence homologous to a genomic DNA sequence of the MAPC and ii) one or more non-defective nucleotide sequence(s) corresponding to the defective nucleotide sequence(s);
- (c) culturing the MAPC under conditions sufficient to homologously recombine the exogenous DNA molecule into the genome of the MAPC, wherein the MAPC expresses the functional gene product;
- (d) selecting MAPCs that express the functional gene product; and
- (e) transplanting the MAPCs into the mammal,

10 wherein d) and e) can be done in any order or simultaneously. For example, MAPCs expressing the functional gene product can be selected *in vitro*, using any selectable marker known in the art, and then transplanted into the mammal. Or, the genetically altered MAPCs can be transplanted into the mammal and the selection can be performed *in vivo*, for example, via antibiotic selection.

15 For purposes of this invention, a "genetic defect", "genetic deficiency" or "defective nucleotide sequence" refer to the presence of at least one mutation, *i.e.*, deletion, addition, or substitution of one or more nucleic acid(s) in a DNA sequence encoding a gene product, including any promoter and regulatory sequences, wherein a functional gene product (nucleic acid and/or protein) is not expressed. A "non-20 defective nucleotide sequence" comprises at least part of a gene which encodes a functional gene product. A "corrected cell" is a cell in which a genetic defect has been corrected by incorporation of a non-defective nucleotide sequence into the cell's genome by homologous recombination.

DNA incorporated into MAPCs can be an entire gene encoding an entire desired product or a gene portion which encodes, for example, the active, functional or defective portion(s) of the gene product. The DNA can be obtained from a source in which it occurs in nature or can be produced, using genetic engineering techniques or synthetic processes. After transfection into MAPCs, the non-defective nucleotide sequence is stably incorporated into the cell's genome.

Tissue specific expression of the gene product can occur, as the homologously recombined nucleotide sequence will be under the control of the endogenous gene promoter by virtue of its locus of integration. For example, a sequence encoding insulin would be produced in islet cells, hemoglobin would be produced in erythroid progenitor cells, erythropoietin in kidney epithelial cells and liver hepatocytes, etc.

A vector, more specifically, a correction vector, which includes the non-defective nucleotide sequence and additional sequences, such as sequences necessary for expression and/or integration of the nucleotide sequence, can be used. Any type of vector known in the art may be used, including, but not limited to, retroviral vectors, adenoviral vectors, adeno-associated viral vectors, other DNA virus-based vectors (such as herpes simplex virus), and non-viral plasmid vectors. In a preferred embodiment, the vector is a plasmid.

The total length of the DNA molecule in the correction vector will vary according to the number of components (non-defective nucleotide sequence, selectable marker gene, etc.) and the length of each. It will also vary depending upon the nature of the genetic defect in a given individual. The nature of the genetic defect can routinely be determined by the skilled artisan using methods known in the art. Once the nature of the genetic defect is known, vectors can be designed based on the site and the extent of the mutation in an individual. In general, a correction vector can include a non-defective version of the defective nucleotide sequence, along with homologies to sequences upstream and/or downstream of the defective sequence. The length of the donor non-defective sequence varies with the specific genetic defect. As small as a single nucleotide mutation may cause a genetic defect. Therefore, the length of the donor DNA used to correct the defect can be decided based on the preferences of choice and design. For example, to correct a single nucleotide mutation, a donor fragment should at least include the correct nucleotide

corresponding to the mutated or defective nucleic acid. For example, a donor fragment of 10-20 base pairs spanning the 5' and 3' region surrounding the mutation can be employed. Yet, an even larger or smaller fragment could be used in a given situation. However, generally the length of the donor DNA is at least about 20 nucleotides. Preferably, the length is between about 20 and 500 nucleotides. The length of the upstream and/or downstream homologous sequences (the targeting DNA) is preferably at least about 1000 nucleotides, more preferably between about 2000 to about 3,000 nucleotides, and most preferably between about 3000 to about 5,000 nucleotides, but again, its actual length in a given situation can be decided based on experimental choice and design. The goal of the design of the DNA molecule is to have sufficient homology with genomic DNA to undergo homologous recombination, and thus it serves to target integration into genomic DNA such that additional targeting sequences are unnecessary.

Stably transfected cells are selected and cultured to propagate a sufficient 15 population of cells for transplantation. Recombinant MAPCs can be administered to a subject by a variety of methods known in the art. Preferably, administration is through injection, including but not limited to, transvascular injection, intramuscular injection, and intravenous injection.

Intravenous injection is the simplest method of cell administration, however 20 a greater degree of dependence on homing of the MAPCs is required for them to reach a tissue of interest. Hence, if necessary, the MAPCs can be directly injected (or otherwise transplanted) into the tissue(s) of interest. Carefully controlled dosing, which is readily determined by one skilled in the art, enhances this method of administration.

25 A method to potentially increase cell survival is to incorporate recombinant MAPCs into a biopolymer or synthetic polymer. Depending on the patient's condition, the site of injection might prove inhospitable for cell seeding and growth because of scarring or other impediments. Examples of biopolymer include, but are not limited to, cells mixed with fibronectin, fibin, fibrinogen, thrombin, collagen, 30 and proteoglycans. This could be constructed with or without included cytokines or differentiation factors. Additionally, these could be in suspension but residence time at sites subjected to flow would be nominal. Another alternative is a three-dimension gel with cells entrapped within the interstices of the cell biopolymer

admixture. Again differentiation factors or cytokines could be included with the cells. These could be deployed by injection via various routes described herein, via cardiac catheters, or other surgical procedures.

An issue concerning the therapeutic use of stem cells, in general, is the quantity of cells necessary to achieve an optimal effect. Preferably, approximately 1  $\times 10^6$  recombinant MAPCs are transplanted into the mammal. In current human studies of autologous mononuclear bone marrow cells, empirical doses ranging from 1 to 4  $\times 10^7$  cells have been used with encouraging results. However, different scenarios may require optimization of the amount of cells injected into a tissue of interest. Thus, the quantity of cells to be administered will vary for the subject being treated. The precise determination of what would be considered an effective dose may be based on factors individual to each patient, including their size, age, size of the infarct or other tissue damage, and amount of time since the damage occurred. Therefore, dosages can be readily ascertained by those skilled in the art from this disclosure and the knowledge in the art.

The skilled artisan can readily determine the amount of cells and optional additives, vehicles, and/or carrier in compositions and to be administered in methods of the invention. Typically, any additives (in addition to the active stem cell(s) and/or cytokine(s)) are present in an amount of 0.001 to 50 wt % solution in phosphate buffered saline, and the active ingredient is present in the order of micrograms to milligrams, such as about 0.0001 to about 5 wt %, preferably about 0.0001 to about 1 wt %, most preferably about 0.0001 to about 0.05 wt % or about 0.001 to about 20 wt %, preferably about 0.01 to about 10 wt %, and most preferably about 0.05 to about 5 wt %. Of course, for any composition to be administered to an animal or human, and for any particular method of administration, it is preferred to determine therefore: toxicity, such as by determining the lethal dose (LD) and LD<sub>50</sub> in a suitable animal model e.g., rodent such as mouse; and, the dosage of the composition(s), concentration of components therein and timing of administering the composition(s), which elicit a suitable response. Such determinations are routine in the art and can be readily ascertained from the present disclosure and the documents cited herein. Similarly, the timing of sequential administrations can be routinely ascertained by the skilled artisans.

When administering a therapeutic composition of the present invention, it will generally be formulated in a unit dosage injectable form (solution, suspension, emulsion). The pharmaceutical formulations suitable for injection include sterile aqueous solutions and dispersions. The carrier can be a solvent or dispersing medium containing, for example, water, saline, phosphate buffered saline, polyol (for example, glycerol, propylene glycol, liquid polyethylene glycol, and the like) and suitable mixtures thereof.

Additionally, various additives which enhance the stability, sterility, and isotonicity of the compositions, including antimicrobial preservatives, antioxidants, chelating agents, and buffers, can be added. Prevention of the action of microorganisms can be ensured by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, and the like. In many cases, it will be desirable to include isotonic agents, for example, sugars, sodium chloride, and the like. Prolonged absorption of the injectable pharmaceutical form can be brought about by the use of agents delaying absorption, for example, aluminum monostearate and gelatin. According to the present invention, however, any vehicle, diluent, or additive used is compatible with the cells and readily ascertained by the skilled artisans.

Sterile injectable solutions (and suspensions) can be prepared by incorporating the cells utilized in practicing the present invention in the required amount of the appropriate liquid preparation, optionally containing various amounts of other ingredients, as desired.

In one embodiment, recombinant MAPCs can be administered initially, and thereafter maintained by further administration of recombinant MAPCs. For instance, recombinant MAPCs can be administered by one method of injection, and thereafter further administered by a different or the same type of method. The patient's levels can then be maintained, for example, by intravenous injection, although other forms of administration, dependent upon the patient's condition, can be used.

It is noted that human subjects are treated generally longer than experimental animals, such that treatment has a length proportional to the length of the disease process and effectiveness. The doses may be single doses or multiple doses over a period of several days, weeks, months, years, or for the life of the recipient. Thus,

one of skill in the art can routinely scale up from animal experiments, e.g., rats, mice, and the like, to humans, by techniques gleaned from this disclosure and documents cited herein and the knowledge in the art. The treatment generally has a length proportional to the length of the disease process and drug effectiveness and the subject being treated.

Examples of compositions comprising recombinant MAPCs include liquid preparations for administration, including suspensions; and, preparations for intramuscular or intravenous administration (e.g., injectable administration), such as sterile suspensions or emulsions. Such compositions may be in admixture with a suitable carrier, diluent, or excipient such as sterile water, physiological saline, glucose, dextrose, or the like. The compositions can also be lyophilized. The compositions can contain auxiliary substances such as wetting or emulsifying agents, pH buffering agents, gelling or viscosity enhancing additives, preservatives, flavoring agents, colors, and the like, depending upon the route of administration and the preparation desired. Standard texts, such as "REMINGTON'S PHARMACEUTICAL SCIENCE", 17th edition, 1985, incorporated herein by reference, can be consulted to routinely prepare suitable preparations.

Compositions of the invention are conveniently provided as liquid preparations, e.g., isotonic aqueous solutions, suspensions, emulsions or viscous compositions, which may be buffered to a selected pH. Liquid preparations are normally easier to prepare than gels, other viscous compositions, and solid compositions. Additionally, liquid compositions are somewhat more convenient to administer, especially by injection. Viscous compositions, on the other hand, can be formulated within the appropriate viscosity range to provide longer contact periods with specific tissues.

The choice of suitable carriers and other additives will depend on the exact route of administration and the nature of the particular dosage form, e.g., liquid dosage form (e.g., whether the composition is to be formulated into a solution, a suspension, gel or another liquid form, such as a time release form or liquid-filled form).

Solutions, suspensions and gels normally contain a major amount of water (preferably purified, sterilized water) in addition to the cells. Minor amounts of other ingredients such as pH adjusters (e.g., a base such as NaOH), emulsifiers or

dispersing agents, buffering agents, preservatives, wetting agents and jelling agents (e.g., methylcellulose), may also be present. The compositions can be isotonic, i.e., they can have the same osmotic pressure as blood and lacrimal fluid.

The desired isotonicity of the compositions of this invention may be

5. accomplished using sodium chloride, or other pharmaceutically acceptable agents such as dextrose, boric acid, sodium tartrate, propylene glycol or other inorganic or organic solutes. Sodium chloride is preferred particularly for buffers containing sodium ions.

Viscosity of the compositions, if desired, can be maintained at the selected

10. level using a pharmaceutically acceptable thickening agent. Methylcellulose is preferred because it is readily and economically available and is easy to work with. Other suitable thickening agents include, for example, xanthan gum, carboxymethyl cellulose, hydroxypropyl cellulose, carboomer, and the like. The preferred concentration of the thickener will depend upon the agent selected. The important 15. point is to use an amount, which will achieve the selected viscosity. Viscous compositions are normally prepared from solutions by the addition of such thickening agents.

A pharmaceutically acceptable preservative or cell stabilizer can be employed to increase the life of the compositions. Preferably, if preservatives are 20. necessary, it is well within the purview of the skilled artisan to select compositions that will not affect the viability or efficacy of the MAPCs as described in the present invention.

Those skilled in the art will recognize that the components of the compositions should be selected to be chemically inert. This will present no 25. problem to those skilled in chemical and pharmaceutical principles, or problems can be readily avoided by reference to standard texts or by routine experiments gleaned from the present disclosure and the documents cited herein.

The genetic defect corrected by methods of the invention can be at any known locus of a gene, whereby a functional gene product is not expressed. The preferred genes for various applications of the present invention encode FANCA, FANCB, FANCC, FANCD1, FANCD2, FANCE, FANCF, FANCG, hemoglobin, factor VIII, factor IX, erythropoietin, CFTR, collagen I, alpha 1 anti-trypsin, gamma-C, Rag1, Rag2, adenosine deaminase, phenylalanine hydroxylase, fumarate

dehydroxylase, LDL receptor, aL-iduronidase or b-glucuronidase. More preferably, the gene encodes FANCC.

A specific example of the practical application of the patent invention is in treatment of Fanconi Anemia ("FA"). FA is an autosomal, recessive disorder. The clinical hallmark of FA is bone marrow failure. Such a deficiency in blood cell types is known to occur due to the progressive loss of hematopoietic progenitor cells (Fanconi, 1927). Congenital abnormalities and predisposition to several forms of cancer, such as acute myelogenous leukemia and squamous cell carcinoma also characterize patients suffering from this disease.

At present, the only mode of therapy to treat FA is blood marrow transplant. Recently, gene therapy efforts have been initiated by transfection of the intact gene into FA donor cells using viral vectors. However, there are several drawbacks to viral vectors that make them undesirable for therapeutic use. Methods of the present invention repair the defective gene *in situ*, yielding normal regulated gene expression and avoiding the random insertion events involved in other gene therapy methods.

Cells that are FANCC-/- can be selected by treatment of the cells with a dose of mitomycin C that is toxic to cells that do not express the gene product and non-toxic to cells that express the gene product. The preferred dose of mitomycin C is about 10 ng/ml for about 48 hours. Alternatively, FANCC-/- cells can be selected by treatment of the cells with a dose of cyclophosphamide and a dose of irradiation at levels that are toxic to cells that do not express the gene product and non-toxic to cells that express the gene product.

In one embodiment, the correction vector can comprise a gene encoding a selectable marker which confers a selectable phenotype, such as drug resistance, nutritional auxotrophy, resistance to a cytotoxic agent or expression of a surface protein. Selectable marker genes which can be used include, but are not limited to neo, gpt, dhfr, ada, pac, hyg, mdrl and hisD. The selectable marker is preferably an antibiotic resistance gene. The selectable phenotype conferred makes it possible to identify and isolate MAPCs that have received the correction vector containing the selectable marker. Cells that integrate the construct will survive treatment with the selective agent. A subset of the stably transfected cells will be homologously

recombined cells, which can be identified by a variety of techniques, including PCR, Southern hybridization and phenotypic screening.

The selectable marker can optionally be flanked at the 5' and 3' ends by *lox P* sites. In this embodiment, cells are selected based on expression of the selectable marker, and the selectable marker is excised by the Cre enzyme prior to transplantation. The Cre-*lox P* recombination system of bacteriophage P1 has been employed for genetic manipulation of higher eukaryotic cells (Sauer *et al.*, 1988). Cre recombinase recognizes a 34 base pair Cre recombination signal sequence called *lox P*, and can delete genes which are flanked by two *lox P* sites in the same direction. Recombination of *lox P* sites can be achieved by transient transfection of a Cre expression plasmid (Araki *et al.*, 1995) or by stable transfection of an inducible Cre recombinase construct. Alternatively, Cre can be introduced by crosslinking the polypeptide to the HIV trans-activating transduction (Tat) protein and introducing it into the cells. The Tat protein, when fused to a heterologous protein or peptide, can traverse biological membranes in a process called protein transduction (Mann *et al.*, 1991; Anderson *et al.*, 1993; Fawell *et al.*, 1994).

Transformed MAPCs or their progeny are administered to the patient in need thereof, using known methods, via localized injection, including catheter administration, systemic injection, parenteral administration, including subcutaneous and intravascular, oral administration, central nervous system including intrathecal, intrahepatic, intrasplanchic, intraperitoneal (including intraomental), renal, subcapsular, or intramuscular or intrauterine injection into an embryo. Typically, a physician will determine the approximate cell dosage that will be most suitable for an individual subject. The specific dose for any particular subject may be varied and will depend upon a variety of factors including the age, body weight, general health, mode and time of administration and the severity of the particular condition in the subject undergoing transplantation.

Administration can be in conjunction with a pharmaceutically acceptable matrix, which may be biodegradable.

### 30 EXAMPLES

The following examples are provided as a further description of the invention, and to illustrate but not limit the invention.

Example 1Isolation, Culture and Characterization of Mouse MAPCs

Mouse MAPCs (mMAPCs) were isolated, cultured and characterized essentially as described in Furcht et al. (PCT/US00/21387). All tissues were 5 obtained according to guidelines from the University of Minnesota IACUC. Briefly, bone marrow mononuclear cells were obtained using Ficoll-Plaque density gradient centrifugation of bone marrow tissue from 5-6 week old ROSA26, C57/BL6, or FANCC-/- mice.

Alternatively, muscle and brain tissue was obtained from 3-129 day old 10 ROSA26 or C57/BL6 mice. Muscles from the proximal parts of fore and hind limbs were excised and thoroughly minced. The tissue was treated with 0.2% collagenase (Sigma Chemical Co, St Louis, MO) for 1 hour at 37°C, followed by 0.1% trypsin (Invitrogen, Grand Island, NY) for 45 minutes. Cells were then triturated vigorously and passed through a 70-um filter. Cell suspensions were collected and centrifuged 15 for 10 minutes at 1600 rpm. Brain tissue was dissected and minced thoroughly. Cells were dissociated by incubation with 0.1% trypsin and 0.1% DNase (Sigma) for 30 minutes at 37 °C. Cells were then triturated vigorously and passed through a 70-um filter and centrifuged for 10 minutes at 1600 rpm.

Bone marrow mononuclear cells (BMMNC), or muscle or brain cell 20 suspensions, were plated at a density of approximately  $1 \times 10^5$  cells/cm<sup>2</sup> in expansion medium [2% fetal calf serum (FCS) in low glucose Dulbecco's minimal essential medium (LG-DMEM), 10 ng/mL each platelet derived growth factor (PDGF), epidermal growth factor (EGF) and leukemia inhibitory factor (LIF)] and maintained at a cell density of about  $5 \times 10^3$  cells/cm<sup>2</sup>. After 3-4 weeks, cells were recovered by 25 trypsin/EDTA and were depleted of CD45<sup>+</sup>/glycophorin (Gly)-A<sup>+</sup> cells with micromagnetic beads. The resulting CD45/Gly-A<sup>+</sup> cells were replated at 10 cells/well in 96-well plates coated with fibronectin (FN) and were expanded at cell densities between 0.5 and  $1.5 \times 10^3$  cells/cm<sup>2</sup>. The resulting mMAPCs had similar expansion potentials regardless of the tissue from which they were derived. 30 Approximately 1 % of wells seeded with 10 CD45/GlyA<sup>+</sup> cells yielded continuous growing cultures, suggesting that the cells capable of initiating MAPC cultures were rare. mMAPCs were approximately 8-10  $\mu$ m in diameter with a large nucleus and scant cytoplasm. Several populations were cultured for more than 100 population

doublings, and the morphology, phenotype and average telomere length of the cells remained unchanged throughout culture.

mMAPCs derived from bone marrow, muscle and brain were CD13<sup>+</sup>, CD44<sup>+</sup>, CD45<sup>-</sup>, class-I and class-II histocompatibility antigen<sup>+</sup>, Flk1<sup>low</sup> and cKit<sup>+</sup>, as has previously been described for human MAPCs (Furc<sup>h</sup> et al., PCT/US00/21387). As with human cells, mMAPCs cultured on FN expressed transcripts for oct-4, and the LIF-R.

#### Example 2

##### Isolation, Culture and Characterization of Human MAPCs

10 Bone marrow tissue was obtained from healthy volunteer donors (age 2-50 years) after informed consent using guidelines from the University of Minnesota Committee on the use of Human Subject in Research. BMMNCs were obtained by Ficoll-Plaque density gradient centrifugation and depleted of CD45<sup>+</sup> and glycophorin-A<sup>+</sup> cells using micromagnetic beads (Milteny<sup>ii</sup> Biotec, Sunnyvale, CA).

15 Approximately  $5 \times 10^3$  CD45<sup>-</sup>/GlyA<sup>-</sup> cells were diluted in 200  $\mu$ L expansion medium [58% DMEM-LG, 40% MCDB-201 (Sigma Chemical Co, St Louis, MO), supplemented with 1X insulin-transferrin-selenium (ITS), 1X linoleic-acid bovine serum albumin (LA-BSA),  $10^{-8}$  M Dexamethasone,  $10^{-4}$  M ascorbic acid 2-phosphate (all from Sigma), 100 U penicillin and 1,000 U streptomycin (Gibco) and 20 0-10% fetal calf serum (FCS) (Hyclone Laboratories, Logan, UT) with 10 ng/ml of EGF (Sigma) and 10 ng/ml PDGF-BB (R&D Systems, Minneapolis, MN)] and plated in wells of 96 well plates that had been coated with 5 ng/ml of FN (Sigma). Medium was exchanged every 4-6 days. Once wells were >40-50% confluent, adherent cells were detached with 0.25% trypsin-EDTA (Sigma) and replated at 1:4 25 dilution in MAPC expansion medium and in bigger culture vessels coated with 5 ng/ml FN to maintain cell densities between 2 and  $8 \times 10^3$  cells/cm<sup>2</sup>.

Undifferentiated human MAPCs did not express CD31, CD34, CD36, CD44, CD45, CD62-E, CD62-L, CD62-P, HLA-class I and II, cKit, Tie, Tek,  $\alpha_v\beta_3$ , VE-cadherin, vascular cell adhesion molecule (VCAM), or intracellular adhesion molecule (ICAM)-1. MAPCs expressed low/very low levels of  $\beta 2$ -microglobulin,  $\alpha_v\beta_5$ , CDw90, AC133, Flk1 and Flt1, and high levels of CD13 and CD49b.

#### Example 3

##### Differentiation of MAPCs into Multiple Cell Types

The differentiation ability of mouse, rat and human MAPCs was tested by adding differentiation factors (cytokines) that have previously been determined to be involved in the differentiation of ES cells into mesoderm, neuroectoderm, and endoderm. Differentiation required that cells were replated at 1-2 x 10<sup>4</sup> cells/cm<sup>2</sup> in serum free medium, without EGF, PDGF-BB and LIF, but with lineage specific cytokines. Differentiation was determined by RT-PCR, functional studies, and immunohistology for tissue specific markers. The tissue specific markers used were:

- (a) slow twitch myosin and MyoD for muscle,
- (b) von-Willebrand factor (vWF) and Tek for endothelium,
- (c) NF200 and MAP2 for neuroectoderm, and
- (d) cytokeratin-18 and albumin for endoderm

The description below relates to differentiation of bone marrow-derived MAPCs. However, mMAPCs derived from muscle or brain were also tested, and could be induced to differentiate to mesoderm (endothelial cells), neuroectoderm (astrocytes and neurons) and endoderm (hepatocyte-like cells) using the same methods.

#### Neuronal Differentiation

Palmer *et al.* showed that neuroprogenitors can be cultured and expanded with PDGF-BB and induced to differentiate by removal of PDGF and addition of bFGF as a differentiation factor. Based on those studies and studies conducted using hMAPCs, mMAPCs were plated in FN coated wells without PDGF-BB and EGF but with 100 ng/mL bFGF. Progressive maturation of neuron-like cells was seen throughout culture. After 7 days, the majority of cells expressed nestin. After 14 days, 15-20% of MAPCs acquired morphologic and phenotypic characteristics of 25 astrocytes (GFAP<sup>+</sup>), 15-20% of oligodendrocytes (galactocerebroside (GalC)<sup>+</sup>) and 50-60% of neurons (neurofilament-200 (NF-200)<sup>+</sup>). NF200, GFAP or GalC were never found in the same cell, suggesting that it is unlikely that neuron-like cells were hMAPCs or glial cells that inappropriately expressed neuronal markers. Neuron-like cells also expressed Tau, MAP2 and NSE. Approximately 50% of neurons 30 expressed gamma-amino-butyric-acid (GABA) and parvalbumin, 30% tyrosine hydroxylase and dopa-decarboxylase (DDC), and 20% serotonin and tryptophan hydroxylase. Differentiation was similar when MAPCs had been expanded for 40 or >90 population doublings. Quantitative RT-PCR, performed as described in WO

02/064748, confirmed expression of neuroectodermal markers: on day 2 MAPCs expressed otx1 and otx2 mRNA, and after 7 days nestin mRNA was detected.

The effect of fibroblast growth factor (FGF)-8b as a differentiation factor was tested next. This is important *in vivo* for midbrain development and used *in vitro* to induce dopaminergic and serotoninergic neurons from murine ES cells.

When confluent human MAPCs (n=8) were cultured with 10 ng/mL FGF-8b + EGF, differentiation into cells staining positive for neuronal markers but not oligodendrocytes and astrocytes was seen. Cells had characteristics of GABAergic (GABA<sup>+</sup>; 40±4%), dopaminergic (DOPA, TH, DCC and DTP<sup>+</sup>, 26±5%) and serotoninergic (TrH, serotonin and serotonin-transporter<sup>+</sup>, 34±6%) neurons. DOPA<sup>+</sup> neurons stained with antibodies against Nurr1, suggesting differentiation into midbrain DA neurons.

FGF-8b induced cells did not have the electrophysiological characteristics of mature neurons. Therefore, cells from 3-week old FGF-8b supported cultures were co-cultured with the glioblastoma cell line U-87, in the presence of FGF-8b, for an additional 2-3 weeks. Cells then acquired a more mature neuronal morphology, having increased cell size, cell number, and length and complexity of neurites. Furthermore, the cells acquired electrophysiological characteristics of mature neurons, *i.e.* a transient inward current, blocked reversibly by 1  $\mu$ M tetrodotoxin (TTX), and having a time course and voltage-dependence typical of the type of voltage-activated sodium current found in mature neurons.

When human MAPCs (n=13) were cultured with 10 ng/ml brain-derived neurotrophic factor (BDNF) + EGF, differentiation was to exclusively DOPA, TH, DCC, DTP and Nurr1 positive neurons. Although BDNF supports neural differentiation from ES cells and neural stem cells (NSCs) (Peault, 1996; Choi *et al.* 1998), no studies have shown exclusive differentiation to DA-like neurons. Similar results were seen for mouse MAPCs induced with bFGF and for rat MAPCs induced with bFGF and BDNF.

#### Endothelial Differentiation

As an example of mesoderm, differentiation was induced to endothelium. Undifferentiated mouse MAPCs did not express the endothelial markers CD31, CD62E, Tek or vWF, but expressed low levels of Flk1. Mouse MAPCs were cultured in FN-coated wells with 10 ng/mL of the endothelial differentiation factor

VEGF-B. Following treatment with VEGF for 14 days, >90% of MAPCs, irrespective of the number of population doublings they had undergone, expressed Flt1, CD31, vWF or CD62, consistent with endothelial differentiation. Like primary endothelial cells, MAPC-derived endothelial cells formed vascular tubes within 6 hours after replating in Matrigel™.

Similarly, human MAPCs express Flk1 and Flt1 but not CD34, Muc18 (P1H12), PECAM, E- and P-selectin, CD36, or Tie/Tek. When human MAPCs (2 x 10<sup>4</sup> cells/cm<sup>2</sup>) were cultured in serum free medium with 20 ng/mL vascular endothelial growth factor (VEGF), cells expressed CD34, VE-cadherin, VCAM and Muc-18 from day 7 on. On day 14, they also expressed Tie, Tek, Flk1 and Flt1, PECAM, P-selectin and E-selectin, CD36, vWF, and connexin-40. Furthermore, cells could uptake low-density lipoproteins (LDL). Results from the histochemical staining were confirmed by Western blot. To induce vascular tube formation, MAPCs cultured for 14 days with VEGF were replated on Matrigel™ with 10 ng/mL VEGF-B for 6 hours. Endothelial differentiation was not seen when human MAPCs cultured in >2% FCS were used. In addition, when FCS was left in the media during differentiation, no endothelial cells were generated.

At least 1000-fold expansion was obtained when human MAPCs were subcultured, suggesting that endothelial precursors generated from human MAPCs continue to have significant proliferative potential. Cell expansion was even greater when FCS was added to the cultures after day 7.

When human MAPC-derived endothelial cells were administered intravenously (i.V.) in NOD-SCI mice that had a human colon carcinoma implanted under the skin, contribution of the human endothelial cells could be seen to the neovascularization in the tumors. It may therefore be possible to incorporate genetically modified endothelial cells to derive a therapeutic benefit, *i.e.*, to inhibit angiogenesis in cancer, or to promote angiogenesis to enhance vascularization in limbs or other organs such as the heart.

#### Endodermal Differentiation

Mouse MAPC differentiation into endoderm was tested. A number of different culture conditions were tested including culture with the differentiation factors keratinocyte growth factor (KGF), hepatocyte growth factor (HGF) and FGF-4, either on laminin, collagen, FN or Matrigel™ coated wells. When re-plated on

Matrigel™ with 10 ng/mL FGF4 and 10 ng/mL HGF, approximately 70% of MAPCs acquired morphologic and phenotypic characteristics of hepatocyte-like cells. Cells became epithelioid; approximately 10% of cells became binucleated, and about 70% of cells stained positive for albumin, cytokeratin (CK)-18, and HNF-1 $\beta$ .

5 Endodermal-like cells generated in FGF4 and HGF containing cultures also had functional characteristics of hepatocytes, determined by measuring urea levels in supernatants of undifferentiated MAPCs and FGF4 and HGF-induced MAPCs using the Sigma Urea Nitrogen Kit 640 according to the manufacturer's recommendations.

10 No urea was detected in undifferentiated MAPC cultures. Urea production was 10  $\mu$ g/cell/hr 14 days after adding FGF4 and HGF and remained detectable at similar levels until day 25. This is comparable to primary rat hepatocytes grown in monolayer. Presence of albumin together with urea production supports the notion of hepatic differentiation from MAPCs *in vitro*.

15 Given the likely existence of an endodermal lineage precursor cell, MAPC likely give rise to a cell that forms various cells in the liver in the pancreas both exocrine and endocrine components and other endodermal derived cell tissue lineages.

20 Hematopoietic Differentiation  
The ability of MAPCs to differentiate into hematopoietic cells was tested. eGFP transduced human MAPCs that were GlyA, CD45 and CD34 negative (n=20), were co-cultured with the mouse yolk sac mesodermal cell line, YSM5, as suspension cell aggregates for 6 days in serum free medium supplemented with 10 ng/mL bFGF and VEGF. After six days, only eGFP $^{+}$  cells (*i.e.*, MAPC progeny) 25 remained and YSM5 cells had died.

Remaining cells were transferred to methylcellulose cultures containing 10% fetal calf serum supplemented with 10 ng/mL bone morphogenic protein-4 (BMP4), VEGF, bFGF, stem cell factor (SCF), Flt3L, hyper IL6, thrombopoietin (TPO), and erythropoietin (EPO) for 2 weeks. In these cultures, both adherent eGFP $^{+}$  cells and 30 small, round non-adherent cells, which formed many colonies attached to the adherent cells, were detected. The non-adherent and adherent fractions were collected separately and cultured in 10%FCS containing medium with 10 ng/mL VEGF and bFGF for 7 days. Adherent cells stained positive for vWF, formed

vascular tubes when plated on ECM, and were able to uptake a-LDL, indicating their endothelial nature. 5-50% of the non-adherent cells stained positive for human specific GlyA and HLA-class I by flow cytometry. Gly-A<sup>+</sup>/HLA-class-I<sup>+</sup> cells were selected by FACS. On Wright-Giemsa, these cells exhibited the characteristic morphology and staining pattern of primitive erythroblasts. Cells were benzidine and human hemoglobin (Hb) positive. By RT-PCR it was determined that these cells expressed human specific Hb-e, but not Hb-a.

When replated in methylcellulose assay with 20%FCS and EPO, small erythroid colonies were seen after 10 days, and 100% of these colonies stained positive for human specific GlyA and Hb. As selection of MAPCs depends on the depletion of CD45<sup>+</sup> and Gly A<sup>+</sup> cells from bone marrow, and cultured MAPCs were CD45<sup>-</sup> and GlyA<sup>-</sup> at all times examined, using both FACS and cDNA array analysis, it is very unlikely that these results were due to contamination of MAPCs with hematopoietic cells.

15

#### Example 4

##### Homing and Engraftment of Mammalian MAPCs into Numerous Organs in the Body

mMAPCs were tested to determine whether they had the ability to engraft and differentiate *in vivo* into tissue specific cells. mMAPCs were grown as described in Example 1 from a LacZ transgenic C57 Black 6, ROSA 26 mouse. 10<sup>6</sup> mMAPCs from the LacZ mouse were injected intravenously into NOD-SCID mice tail veins, with or without 250 Rads of total body radiation 4-6 hrs prior to the injection. The animals were sacrificed by cervical dislocation at 4-24 weeks after the injections.

25 

#### Tissue Harvest

*Blood and bone marrow:* 0.5-1 ml of blood was obtained at the time animals were sacrificed. Bone marrow was collected by flushing femurs and tibias. For phenotyping, red cells in blood and bone marrow were depleted using ice cold ammonium chloride (Stem Cell Technologies Inc., Vancouver, Canada) and 10<sup>5</sup> cells were used for cytospin centrifugation. For serial transplantation, 5x10<sup>7</sup> cells from 2 femurs and 2 tibias were transplanted into individual secondary recipients via tail vein injection. Secondary recipients were sacrificed after 7-10 weeks.

*Solid organs:* Lungs were inflated with 1 ml 1:4 dilution of OCT compound (Sakura-Finetek Inc, USA) in PBS. Specimens of spleen, liver, lung, intestine, skeletal muscle, myocardium, kidney and brain of the recipient animals were harvested and cryopreserved in OCT at -80°C and in RNA Later (Ambion Inc., Austin, TX, USA) at -20°C for quantitative PCR.

**mMAPCs Engraft and Differentiate Into Tissue Specific Cells *In Vivo***

Engraftment of the  $\beta$ -gal/neomycin (NEO) transgene-containing cells (Zambrowicz *et al.*, 1997) was tested by immunohistochemistry for  $\beta$ -gal and by quantitative-PCR for the NEO gene. Immunohistochemistry as described in WO 10 02/064748.

Engraftment, defined as detection of >1%  $\beta$ -gal positive cells, was seen in hematopoietic tissues (blood, bone marrow and spleen) as well as epithelium of lung, liver, and intestine of all recipient animals tested as shown in Table 1 below.

**Table 1**

| Animal | Time<br>(Weeks) | Engraftment levels (%) determined by immunofluorescence or (quantitative -PCR) |        |       |          |         |           |           |
|--------|-----------------|--|--------|-------|----------|---------|-----------|-----------|
|        |                 | Radiation  | Marrow | Blood | Spleen   | Liver   | Lung      | Intestine |
| 1      | 4               | No   | 2 (1)  | 2     | 5        | 7       | 4         | 2         |
| 2      | 5               | No   | 3 (4)  | 4     | 5        | 9       | 5         | 3         |
| 3      | 10              | No   | 1      | 3     | 3        | 6       | 3         | 2         |
| 4      | 16              | No   | 4      | 2     | 3        | 4       | 3         | 4 (4.9)   |
| 5      | 24              | No   | 3      | 2     | 3        | 6       | 4         | 1         |
| 6      | 8               | Yes  | 8 (8)  | 6     | 4        | 5       | 2 (1.1)   | 7         |
| 7      | 8               | Yes  | 10     | 8     | 7 (7.3)  | 4       | 6         | 8         |
| 8      | 8               | Yes  | 5      | 8     | 3        | 5       | 5         | 6         |
| 9      | 8               | Yes  | 7      | 5     | 5        | 6       | 4         | 6         |
| 10     | 10              | Yes  | 5 (6)  | 7     | 9 (12.5) | 5       | 2         | 8         |
| 11     | 11              | Yes  | 8      | 8     | 6        | 5       | 3         | 10 (11.9) |
| 12     | 11              | Yes  | 6      | 5     | 4        | 8 (6.2) | 10 (12.3) | 8         |
| SR-1   | 7               | Yes  | 6      | 7     | 5        | 1 (1.7) | 5         | 8         |
| SR-2   | 10              | Yes  | 5      | 4     | 8        | 3       | 4         | 6         |

These results show that mammalian MAPCs can be purified, expanded *ex vivo*, infused I.V., and can home to various sites in the body, engraft into numerous organs, and that the cells are alive in these various organs one month or longer. Such donor cells, and undifferentiated and differentiated progeny of them, are found in organs including, but not limited to, bone marrow, spleen, liver and lung.

MAPCs can thus be used to repopulate one or more compartment(s), or to augment or restore cell or organ function.

Example 5

MAPCs Can Be Effectively Transfected

5 In order for MAPCs to be successfully used in gene targeting/gene correction applications, the cells must be amenable to transfection. Several experiments were performed to determine a) whether MAPCs can be transfected and b) if so, which transfection methods are most effective. All transfections were performed using a mammalian green fluorescent protein (GFP) expression vector pEGFP-N1 (Clontech). In each case, 10 ug of DNA was transfected into  $0.3 \times 10^6$  cells. Expression of GFP was determined 2 days after transfection by visualizing cells using a fluorescence microscope (excitation wavelength of approximately 488 nm) and counting the % of cells displaying green fluorescence. The transfection methods used were:

10 (a) Calcium phosphate precipitation (CalPhos kit; Clontech);  
(b) Effectene<sup>TM</sup> (Qiagen): according to manufacturer's instructions;  
(c) DMRIE-C (Invitrogen): according to manufacturer's instructions;  
(d) AVET (Bender Med Systems);  
(e) AVET + DMRIE-C: preincubation of the AVET-DNA complex with  
20 the DMRIE-C transfection reagent for 30 minutes at 37°C followed by overnight incubation with cells to be transfected;  
(f) Superfect<sup>TM</sup> (Gibco-BRL): according to manufacturer's instructions;  
(g) electroporation; and  
(h) nucleoporation.

25 Most of the known methods of transfection tested were found to be sub-optimal in MAPCs, owing to the requirement that they must be grown at a low cell density of about 500 cells/cm<sup>2</sup> in order to remain undifferentiated. Of the transfection methods that were tried, Superfect, a liposome mediated method, electroporation, and nucleoporation gave the best results, having a significant  
30 number of transfectants (Figure 1). The optimized nucleoporation method described herein was the preferred method, yielding a significant percentage of stable transfectants at cell densities low enough to maintain MAPCs in an undifferentiated state.

For transfection with Superfect, MAPCs were seeded at high density, and a transfection rate of 15-18% was achieved at 24 hours post transfection. This mode of transfection requires the cells to be at high density, which is not optimal for MAPCs since close cell-cell contact leads to differentiation of the cells and loss of stem cell-like properties.

After transfection using electroporation at 330V, about 12- 15% MAPCs were positive at 24 hours post transfection (Figure 1 and 2). However, the number of transfected cells decreased to about 5-8% by 48 hours post transfection (Figure 2), and stable transfectants were not observed over a period of 3 weeks in culture.

Nucleoporation, a method of electroporation that uses a combination of special transfection solutions with electric parameters was then performed. A sample of about 200,000 cells or greater was trypsinized and spun down. Media was removed (residual media removed by pipette). It is important to remove all media since volumes can change when excess media is left behind. Nucleofection solution (100  $\mu$ l) from Amaxa Inc. was added (the Amaxa Kit used for human MSCs is preferred, but comparable results can be obtained with mES kit and Kit V). Cells were pipetted up and down gently to form a uniform suspension. DNA was added up to a maximum concentration of 60  $\mu$ g DNA per ml. The suspension was mixed and transferred to a 4 mm electrode gap cuvette (supplied by Amaxa Inc.) without introducing air bubbles. The cuvette was placed in the cuvette holder, and the nucleoporation was set to Program A-23 (also supplied by Amaxa Inc.).

After nucleoporation, the cuvette was removed from the holder. The cells were retrieved using plastic pipettes and transferred to eppendorf tubes containing 1 ml MAPC media pre-warmed to 37°C. The cells were stabilized at 37 °C for 5-10 minutes. The transfected MAPCs were then spun down using a desktop centrifuge and plated on FN-coated plates. Alternatively, cells can be plated directly onto FN-coated plates without centrifugation. Assuming 75% cell death, cells were plated at appropriate density. Media was replaced after 12-16 hours and cells were monitored for gene expression.

While different nucleofection solutions (Amaxa Inc.) showed a minor variation in the transfection efficiency, the Program A-23 yield was as high as 25% transfection in mouse MAPCs at 24 h post transfection, and nearly 35% at 48 h post transfection (Figure 2). This result was much higher than the efficiencies obtained

with Superfect or electroporation. Further, stable cells that were resistant to the drug hygromycin were generated following transfection with a plasmid carrying this resistance gene. This indicates that the program and nucleoporation solution were not toxic to MAPCs. Similar results were obtained with human MAPCs (10-14% transfection efficiency) and rat MAPCs (over 30% transfection efficiency).

With this approach, MAPCs only need to be kept at high density transiently, during a course of nucleoporation that does not exceed 5 minutes. Therefore, the cells are not exposed to high cell density conditions for a long period, and hence can be maintained in an undifferentiated state.

10

#### Example 6

##### Gene Targeting Can Be Effectively Achieved in MAPCs

In order to test whether MAPCs are susceptible to gene targeting by homologous recombination, the hypoxanthine phosphoribosyl transferase (HPRT) gene was targeted. HPRT catalyzes the synthesis of inosinate, a precursor of AMP and GMP. Cells expressing functional HPRT can grow in media containing hypoxanthine, aminopterin and thymidine (HAT media). Conversely, cells expressing functional HPRT are sensitive to media containing 6-thioguanine (6-TG) and cannot grow in it.

The HPRT locus was targeted using the vector illustrated in Figure 3. This vector contains sequence from exons II and III of the HPRT gene, but has a hygromycin resistance ( $\text{Hyg}^R$ ) gene inserted within the exon II sequence. Thus, successful homologous recombination of the targeting vector with the wild type HPRT gene results in expression of a truncated, non-functional HPRT gene product, and cells become 6-TG resistant ( $6\text{-HT}^R$ ). In some cells random integration of the targeting vector may occur. However, these cells can be readily distinguished from those cells in which correct targeting of the HPRT locus has been achieved, as only those cells where the HPRT locus has been disrupted (*i.e.* by homologous recombination of the targeting vector) will be 6-TG resistant. All cells containing the vector will be hygromycin resistant, regardless of where in the genome it is integrated.

MAPCs were grown in HAT media for 3-5 days, seeded, and cultured for 12-16 hours as described in Example 2. The current protocols in the art for gene targeting needed to be modified to accommodate MAPCs. MAPCs were maintained

at low density during all steps of the procedure, including during drug selection (where corrected cells are generally picked as colonies). In contrast to other protocols known in the art, MAPCs were seeded in 96 well plates, and targeted clones were picked and maintained at low density.

5 The cells were transfected with the HPRT targeting vector pHPRThyg (provided by Andrew Porter, MRC, London; Yanez and Porter ACG (1999) Gene Therapy 6:1282-1290), linearized with the restriction endonuclease Sal I, using Superfect<sup>TM</sup> according to the manufacturer's instructions (3 hour treatment). Cells were then trypsinized, seeded at normal MAPC plating density, and grown for 2  
 10 days. Cells were grown for three more days in the presence of 120 mg/ml hygromycin to select successfully transfected cells. Fresh media containing hygromycin (120 mg/ml) and 6-HT (15 mg/ml) was added to the cells and they were cultured for 7 days.

This targeting experiment was repeated on two separate occasions. The  
 15 results are illustrated in Table 2 and in Figure 4, and show that MAPCs can be successfully targeted at the HPRT locus. Furthermore, the results shown in Figure 4 illustrate that the level of targeting that can be achieved using MAPCs is even higher than that described previously by Hatada et al. (2001) for ES cells and HSCs.

Table 2

|              | Number of Hyg <sup>R</sup> cells | Number of 6TG <sup>R</sup> cells | Gene Targeting Frequency (x10 <sup>-5</sup> ) |
|--------------|----------------------------------|----------------------------------|---|
| Experiment 1 | 44,000                           | 5                                | 12  |
| Experiment 2 | 37,000                           | 5                                | 13.5  |

20

Example 7Gene Correction and Phenotype Reversal In Mouse Model of Fanconi Anemia

Fanconi Anemia (FA) is an autosomal, recessive disorder resulting in congenital abnormalities, bone marrow failure and predisposition to several forms of cancer. At present the only mode of therapy to treat FA is bone marrow transplant.  
 25 Recently, gene therapy efforts have been initiated involving transfection of the intact gene into FA donor cells using viral vectors. However, such methods are plagued by random insertion events. An ideal solution would be to repair the defective gene itself, thereby yielding naturally regulated FA protein expression. The methods of the present invention can be used to deliver to animals genetically corrected MAPCs

that produce clinically relevant levels of FA protein, and can thus be used in the treatment for FA.

Mice with disrupted FA genes have been created. For example, Whitney *et al.* (1996) generated a mouse in which expression of the FA complementation group C gene (FANCC) was disrupted. This FANCC-/- "knock out" mouse has a neomycin cassette replacing exon 9 of the gene, which results in a frame-shift that leads to the formation of a truncated, non-functional FANCC protein. These mice display many of the characteristics of human FA, for example hypogonadism and reduced fertility and development of pancytopenia (resulting from marrow aplasia) upon exposure to mitomycin C.

The methods of the present invention can be used to correct the genetic and phenotypic defects in the above FANCC-/- knockout mice, as illustrated in Figure 5, and as described in this example.

#### Isolation of MAPCs from the FANCC-/- Mouse.

MAPCs are generally isolated from bone marrow cells. Because the bone marrow of some Fanconi patients may be defective, MAPCs are particularly well-suited for use in these patients because they can be isolated from tissues other than bone marrow. In the case of FANCC-/- mice, muscle and heart cells were also used to generate MAPCs. Brain can also be used for isolation of MAPCs, as is discussed in Example 1. Isolated cells were plated in MAPC expansion medium, consisting of DMEM-LG (58%, Gibco-BRL, Grand Island, NY), MCDB-201 (40%, Sigma Chemicals Co, St Louis, MO), 2% FCS (Hyclone Laboratories, Logan, UT) supplemented with 1X insulin-transferrin-selenium (ITS), 1X linoleic acid-bovine serum albumin (LA-BSA),  $10^{-8}$  M dexamethasone,  $10^{-4}$  M ascorbic acid 2-phosphate (AA), 100U penicillin and 1,000U streptomycin, in wells of a 6 well plate. Following expansion of cells in culture for about 2-3 weeks, cells were sub cloned at 5 or 10 cells per well in a 96 well plate and growing colonies were chosen and expanded. Four different clones were chosen for the study, of which one of the clones that was 90% diploid and cytogenetically normal was chosen for further characterization.

The morphology of the FANCC-/- cells is spindle shaped, with an average size of 11  $\mu$ m. Normal mouse MAPCs are also spindle shaped, with a size of 10-15

μm. Further, the phenotype of FANCC-/- cells is remarkably similar to that of mouse MAPCs (Table 3).

Table 3

| Markers | MAPC Phenotype | FANCC -/- Bulk n = 4 | C#HB2 n = 14  |
|---------|----------------|----------------------|---------------|
| CD45    | Negative       | Negative             | Negative      |
| CD31    | N.A.           | < 1% Positive        | Negative      |
| CD34    | Negative       | Negative             | Negative      |
| CD44    | Negative       | Negative             | Negative      |
| Sca-1   | Positive – dim | ~50% Positive        | ~90% Positive |
| Gr-1    | N.A.           | Negative             | Negative      |
| Thy-1   | Negative       | ~50% Positive        | <10% Positive |
| Flk-1   | Negative       | Negative             | Negative      |
| cKit    | N.A.           | Negative             | Negative      |

5 In order to demonstrate that the FANCC-/- cells are indeed multipotent, cells were seeded for differentiation into multiple lineages. Figure 6 shows morphological changes of FANCC-/- cells following exposure to appropriate cytokines, as described in Example 3, for 11 days in culture.

Correction of the FANCC gene by Homologous Recombination.

10 Once MAPCs have been isolated as described above, a correction vector can be used to replace the defective segment of the gene by homologous recombination. This correction vector (Figure 7) can be made as follows: Vector p7A, described by Whitney *et al.* (1996) comprises a 13.4 kb BglII/NheI fragment derived from a lambda clone and spanning exons 8, 9, and 10 of the mouse FANCC gene, cloned into the commercially available pBluscript<sup>R</sup> cloning vector (Stratagene Inc.). A fragment spanning the disrupted region of the mouse FANCC gene in the FANCC-/- knockout, is excised from p7A and subcloned into any suitable mammalian vector that has an appropriate drug resistance marker. In the present example, the AgeI/ApaBI sites (within the BglII/NheI fragment) are used to subclone approximately 10217 bp of sequence spanning exons 8 and 9 of the mouse FANCC gene, into the mammalian expression pREP4, which has hyg resistance marker, to generate the final correction vector. The AgeI/ApaBI fragment in the correction

vector has 4266 bp of sequence upstream of the 2498 bp disruption in the FANCC-/- knockout, and 3403 bp of downstream sequence, thus providing sufficient homology to promote gene targeting by homologous recombination. The total length of the correction vector is 10217 bp.

5 Mouse FANCC-/- MAPCs are transfected with the correction vector by the methods described in Example 5. Alternatively, MAPCs may be transfected immediately after bone marrow harvesting. In those cells where homologous recombination spontaneously occurs, the neo selection cassette is replaced with the natural exon 9 sequence from the mouse FANCC gene. Thus, corrected cells are not 10 neomycin resistant and cannot grow in media containing neomycin or G418. Corrected cells are selected by exposing the MAPCs to 10 ng/ml mitomycin C for about 48 hours.

Characterization of the Corrected Cells.

The correction of the FANCC gene is confirmed using RT-PCR analysis.

15 The predominant cellular phenotype of FA is chromosomal instability and hypersensitivity to DNA cross linking agents such as mitomycin C and cyclophosphamide (Sasaki & Tonomura, 1973; Auerbach & Wolman, 1976; Ishida & Buchwald, 1982). Therefore, a reduction in sensitivity to mitomycin C and/or cyclophosphamide, and a reduction in chromosomal instability, are used as measure 20 of phenotypic correction of MAPCs.

(a) RT PCR analysis.

Total cytoplasmic RNA is isolated from FANCC-/-, corrected and wild type MAPCs. Reverse transcription with random hexamer primers is performed to generate a cDNA copy of the mRNA, using standard procedures known in the art.

25 This cDNA is then used as the template in PCR reactions. The forward primer has sequence from exon 8 of the FANCC gene (5'-CTGCCAACCTGCCATCTTCAG-3')(SEQ ID NO:3) and the reverse primers have sequences from exon 9 (5'-AAGAGCAGCTAGTACTTCTGG-3')(SEQ ID NO:4) or exon 10 (5'-AGGAAAGTAGGTCTGAGGG-3')(SEQ ID NO:5). PCR products are 30 radioactively labeled by incorporation of <sup>32</sup>P dATP into the PCR reaction mixture. PCR products are separated on a 5% polyacrylamide gel and the gel is exposed to a phosphorimager screen overnight. Confirmation of the correction of the FANCC gene is obtained by comparison of the PCR product bands in the presumed corrected

and wild type MAPCs. The major PCR product in MAPCs that have been corrected is the same size as that found in wild-type MAPCs, but differs in size from that found in uncorrected FANCC-/- MAPCs.

(b) Cell cycle analysis

FA cells are known to arrest at the G2 phase of the cell cycle following treatment with mitomycin C (Heinrich *et al.*, 1998), therefore functional correction of the FANCC-/- phenotype can be tested by determining if mitomycin C causes G2 arrest. FANCC-/-, corrected and wild type MAPCs, are treated with 10 ng/ml mitomycin C for 48 hours. Approximately  $1 \times 10^6$  MAPCs are then fixed in 70% ethanol overnight, digested with RNase A, and stained with propidium iodide according to standard procedures. Following staining, the samples are analyzed for DNA content by flow cytometry using Cell Quest Pro on a FACSCalibur flow cytometry machine, with an excitation wavelength of 488 nm and an emission wavelength of 585 nm. The histogram data generated is analyzed using Modfit software (Verity Software House, Top sham, ME), to determine the cell cycle distribution. By comparing the cell cycle distributions of the presumed corrected cells with those of wild type and FANCC-/- MAPCs, it can readily be determined whether reversal of mitomycin C sensitivity has occurred.

(c) Chromosome breakage analysis.

FA cells are known to exhibit increased chromosomal breakage and radial formation (Auerbach & Wolman, 1976; Auerbach, 1993). FANCC-/- corrected and wild type MAPCs are exposed to various concentrations of clastogens for 24 hours, then treated with colcemid for 3 hours and placed in a hypotonic medium consisting of 25% fetal calf serum and dH<sub>2</sub>O, and fixed to slides. Following staining with Wright's stain, chromosomes are scored for breaks and radials per cell. A level of chromosome breaks and radials similar to that found in wild type MAPCs is indicative of correction of the FANCC-/- phenotype.

Transplantation of the Corrected Cells Into FANCC-/- Mice

The corrected MAPCs are transplanted into FANCC-/- mice by tail vein injection according to established procedures. Approximately,  $1 \times 10^6$  undifferentiated corrected MAPC's are injected into non-irradiated and irradiated 6-9 week old FANCC-/- knockout mice. To confirm the presence of corrected gene, PCR analysis of peripheral blood is carried out 4-6 weeks after transplantation, using

primers specific for the corrected gene. The corrected cells are selected *in vivo* using two mitomycin C dosing regimes: a) an acute dose of 1mg/kg/week and b) a chronic dose of 0.3 mg/kg/week.

#### Reversal of the Phenotype Associated With FANCC Deficiency

5 After the presence of corrected gene is confirmed, 0.3 mg/kg mitomycin C (Calbiochem; La Jolla, CA) is administered to the mice, via intraperitoneal injection, daily for 2-3 weeks. Such administration of mitomycin C is known to cause pancytopenia and bone marrow aplasia in FANCC-/- mice (Carreau *et al.*, 1998). By comparing peripheral blood cell counts between the presumed corrected, 10 FANCC-/- and wild type mice at various time points, reversal of the FANCC-/- phenotype can be confirmed.

#### Example 8

##### Correction of Fanconi Anemia in Humans

Human Fanconi Anemia (FA) is genetically heterogeneous with many 15 mutations reported in each of the complementation group (Strathdee *et al.*, 1992; Joenje *et al.*, 1997, 2000). So far, eight complementation groups: A, B, C, D1, D2, E, F and G (Strathdee *et al.*, 1992; Lo Ten Foe *et al.*, 1996; de Winter *et al.*, 1998; de Winter *et al.*, 2000; Timmers *et al.*, 2001) have been identified. The present 20 example relates to correction of defects in the human FA complementation group C (FANCC) gene. However, the methods of the present invention can equally be used to correct genetic defects in any human gene associated with Fanconi Anemia, or with any other disease or condition caused by known defects in the nucleotide sequence of specific genes.

##### Isolation of MAPC from FA patients

25 Human MAPCs are isolated and cultured as described in Example 2.

##### Correction of the Human FANCC Gene by Homologous Recombination

Correction vectors are made by cloning a region from the "wild type" human FANCC gene that spans the region that is disrupted/mutated in the patient, into a suitable commercially available cloning and/or expression vector, as described in 30 Example 7. Locations and sequences of known mutations in the human FANCC gene, can readily be obtained from genome databases known in the art, such as the Human Gene Mutation Database (HGMD<sup>®</sup>). Correction vectors are made to contain homologous sequence from the wild type FANCC gene, both upstream and

downstream of the disruption or mutation to be corrected. The homologous regions are sufficiently long to allow homologous recombination of the correction vector and the patients' FANCC gene to occur. Correction vectors having homologous regions from 3-5 kb in length can be made and used. Figures 8 and 9 show the sequences of the wild type human FANCC cDNA and protein, respectively. The genomic sequence of the wild type human FANCC gene can be readily obtained from human genome sequence databases known to those of skill in the art.

Human MAPCs derived from the patient are transfected as described in Example 7 with a correction vector. After transfection, the non-defective nucleotide sequence contained in the correction vector homologously recombines with the defective gene in a proportion of the patient's MAPCs, thereby correcting the genetic defect in those cells. Corrected cells are selected by exposing the MAPCs to doses of cyclophosphamide and/or irradiation that are toxic to the defective cells FANCC cells but not toxic to cells expressing the corrected gene (Noll *et al.*, 2001).

**15. Characterization of the Corrected Cells.**

The correction of the FANCC gene in the human MAPCs is confirmed using RT-PCR and/or sequence analysis. The predominant cellular phenotype of FA is chromosomal instability and hypersensitivity to DNA cross linking agents such as mitomycin C and cyclophosphamide (Sasaki & Tonomura, 1973; Auerbach & Wolman, 1976; Ishida & Buchwald, 1982). Therefore, a reduction in sensitivity to mitomycin C and/or cyclophosphamide, and a reduction in chromosomal instability, are also used as measure of phenotypic correction of the human MAPCs.

**(a) RT PCR analysis.**

Total cytoplasmic RNA is isolated from patient's non-corrected MAPCs, 25 corrected MAPCs and wild type "reference" MAPCs derived from individuals not affected with FA. Reverse transcription with random hexamer primers is performed to generate a cDNA copy of the mRNA, using standard procedures known in the art. This cDNA is then used as the template in PCR reactions. Primer sequences based on the human FANCC sequence are chosen according to standard procedures. The 30 forward primer used has sequence from a region of the gene upstream of the genetic defect, and the reverse primer has sequence from a region downstream of the genetic defect. PCR products are radioactively labeled by incorporation of  $^{32}\text{P}$  dATP into the PCR reaction mixture. PCR products are separated on a polyacrylamide gel and

the gel is exposed to a phosphorimager screen overnight. Confirmation of the correction of the patient's FANCC gene is obtained by comparison of the PCR product bands in the presumed corrected and the reference wild type human MAPCs. The major PCR product in MAPCs that have been corrected is the same size as that found in wild-type MAPCs, but differs in size from that found in uncorrected MAPCs originally derived from the patient.

5. (b) Sequence analysis.

Where the genetic defect being corrected is small (for example a point mutation), and therefore can not be distinguished from the wild type version of the gene by differences in the size of PCR products, sequence analysis is performed to check that the patient's corrected cells contain the wild type, as opposed to the defective, sequence. Such sequence analysis is performed by standard techniques known in the art.

10. (c) Cell cycle analysis.

15 FA cells are known to arrest at the G2 phase of the cell cycle following treatment with mitomycin C (Heinrich *et al.*, 1998), therefore functional correction of the patient's FANCC gene defect can be tested by determining if mitomycin C causes G2 arrest in the corrected cells. This is performed as described in example 7.

20. (d) Chromosome breakage analysis.

25 FA cells are known to exhibit increased chromosomal breakage and radial formation (Auerbach & Wolman, 1976; Auerbach, 1993). Therefore, functional correction of the patient's MAPCs is tested by comparing chromosome breakage of the corrected cells, to that in the patient's original defective cells, and wild type "reference cells". This is performed as described in example 7.

25. Transplantation of Corrected Cells

30 Approximately  $1 \times 10^6$  corrected cells are transplanted into the patient intravenously. The exact number of cells and dosage frequency of administration will be determined by the skilled artisan based on the patient's weight, age, sex and severity of the defect.

REFERENCES

Alison, M., & Sarraf, C. (1998). Hepatic stem cells. *J Hepatol* 29: 678-83.

Anderson, D.C. *et al.* (1993). Tumor retention of antibody Fab fragments is enhanced by an attached HIV TAT protein-derived peptide. *Biochem. Biophys Res Commun.* 194:876-884.

Araki, K. *et al.*, (1995). Site-specific recombination of a transgene in fertilized eggs by transient expression of a Cre recombinase. *Proc. Natl. Acad. Sci. U.S.A* 92:160-164

Auerbach, A.D. & Wolman, S.R. (1976). *Nature* 261:494-496

Auerbach, A.D. (1993). *Exp. Hematol.* 21:731-733

Bandyopadhyay, et al., (1999). Nucleotide exchange in genomic DNA of rat hepatocytes using RNA/DNA oligonucleotides. Targeted delivery of liposomes and polyethyleneimine to the asialoglycoprotein receptor. *J Biol Chem.* 274:10163-72.

Caplan, A., *et al.*, U.S. Patent No. 5,486,359

Caplan, A., *et al.*, U.S. Patent No. 5,811,094

Caplan, A., *et al.*, U.S. Patent No. 5,837,539

Carreau, A. *et al.*, (1998). *Blood* 91:2737-2744

Carter, P. J. & Samulski, R. J. (2000). Adeno-associated viral vectors as gene delivery vehicles. *Int. J. Mol. Med.* 6:17-27.

Casey, B. P. & Glazer, P. M. (2001). Gene targeting via triple-helix formation. *Prog. Nucleic Acid Res. Mol. Biol.* 67:163-92.

Cassiede P. *et al.*, (1996). Osteochondrogenic potential of marrow mesenchymal progenitor cells exposed to TGF-beta 1 or PDGF-BB as assayed in vivo and in vitro. *J Bone Miner Res.* 9:1264-73.

Choi, K. (1998). Hemangioblast development and regulation. *Biochem Cell Biol.* 76:947-956.

Choi, K. *et al.*, (1998). A common precursor for hematopoietic and endothelial cells. *Development*. 125:725-732.

Clarke, D. L. *et al.*, (2000). Generalized potential of adult NSCs. *Science* 288:1660-3.

De Winter, J.P. *et al.*, (1998). *Nature Genetics* 20:281-283

De Winter, J.P. *et al.*, (2000). *Hum. Mol. Genet.* 9:2665-22674

De Winter, J.P. *et al.*, (2000). *Nature Genetics* 24:15-16

DiGuisto, *et al.*, U.S. Patent No. 5,681,599

Fanconi (1927). *Jarrb. Kinderh.* 117: 257-280

Fawell, S. *et al.* (1994). Tat-mediated delivery of heterologous proteins into 5 cells. *Proc. Natl. Acad. Sci. USA*. 91:664-668

Fei, R., *et al.*, U.S. Patent No. 5,635,387

Fridenshtein, A. (1982). Stromal bone marrow cells and the hematopoietic microenvironment. *Arkh Patol* 44:3-11.

Furcht *et al.* International Application No. PCT/US00/21387.

Gage, F. H. (2000). Mammalian NSCs. *Science* 287:1433-1438.

Goncz, K. K. *et al.*, (2001). Expression of DeltaF508 CFTR in normal mouse 10 lung after site-specific modification of CFTR sequences by SFHR. *Gene Ther.* 8:961-5.

Gronthos, S. *et al.*, (1994). The STRO-1+ fraction of adult human bone 15 marrow contains the osteogenic precursors. *Blood* 84: 4164-73.

Hatada *et al.* (2001). *Proc. Natl. Acad. Sci.* 97:13807-13811.

Heinrich, M.C. *et al.*, (1998). *Blood* 91; 275-287

Hill, B. *et al.* (1996). High-level expression of a novel epitope of CD59 20 identifies a subset of CD34+ bone marrow cells highly enriched for pluripotent stem cells. *Exp Hematol.* 8:936-43.

Hirata, R. K. & Russell, D. W. (2000). Design and packaging of adeno-associated virus gene targeting vectors. *J. Virol.* 74:4612-20.

Hirata, R. *et al.*, (2002). Targeted transgene insertion into human 25 chromosomes by adeno-associated virus vectors. *Nat. Biotechnol.* 20, 735-8.

Inoue, N. *et al.*, (1999). High-fidelity correction of mutations at multiple chromosomal positions by adeno-associated virus vectors. *J. Virol.* 73:7376-80.

Ishida, R. & Buchwald, M. (1982). *Cancer Research* 42:4000-4006

Jaiswal, N., *et al.*, (1997). *J. Cell Biochem.* 64(2):295-312.

Jarukamjorn, K. *et al.* (1999). Different regulation of the expression of mouse 30 hepatic cytochrome P450 2B enzymes by glucocorticoid and phenobarbital. *Arch Biochem Biophys* 369:89-99.

Jiang, Y. *et al.*, (2002). Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature* 418:41-49.

Jiang, Y. *et al.*, (2002). Multipotent progenitor cells can be isolated from postnatal murine bone marrow, muscle and brain. *Exp. Hematol.* 30:896-904.

Joenje, H. *et al.*, (1997). *Am. J. Hum. Genet.* 61:940-944.

Johnstone, B., Hering, T. M., Caplan, A. I., Goldberg, V. M., Yoo, J. U. (1998). *In vitro* chondrogenesis of bone marrow-derived mesenchymal progenitor cells. *Exp Cell Res.* 241:265-72.

Lo Ten Foe, J.R. *et al.*, (1996). *Nature Genet.* 14:320-323

Mann, D.A. & Frankel, A.D. (1991). Endocytosis and targeting of exogenous HIV-1 Tat protein. *EMBO J.* 10:1733-9.

Martin, G.R. (1981). Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc Natl Acad Sci U.S.A.* 78:7634-8.

Masinovsky, B., U.S. Patent No. 5,837,670

McGlave, *et al.*, U.S. Patent No. 5,460,964

Noll, M. *et al.* (2001). *Mol. Ther.* 3:14023.

Okabe, S., Forsberg-Nilsson, K., Spiro, A. C., Segal, M., and McKay, R. D. (1996). Development of neuronal precursor cells and functional postmitotic neurons from ES cells *in vitro*. *Mech Dev.* 59:89-102.

Orkin, S. (1998). Embryonic stem cells and transgenic mice in the study of hematopoiesis. *Int. J. Dev. Biol.* 42:927-34.

Palmer, T. D., Takahashi, J., and Gage, F. H. (1997). The adult rat hippocampus contains primordial NSCs. *Mol Cell Neurosci.* 18:389-404.

Peault, B. 1996. Hematopoietic stem cell emergence in embryonic life: developmental hematolgy revisited. *J. Hematother.* 5:369.

Pittenger, M. F., Mackay, A. M., Beck, S. C., Jaiswal, R. K., Douglas, R., Mosca, J. D., Moorman, M. A., Simonetti, D. W., Craig, S., and Marshak, D. R. (1999). Multilineage potential of adult human MSCs. *Science* 284:143-147.

Pittenger, M., U.S. Patent No. 5,827,740

Potten, C. (1998). Stem cells in gastrointestinal epithelium: numbers, characteristics and death. *Philos Trans R Soc Lond B Biol Sci* 353:821-30.

Reubinoff, B. E., Pera, M. F., Fong, C. Y., Trounson, A., and Bongso, A. (2000). ES cell lines from human blastocysts: somatic differentiation *in vitro*. *Nat Biotech* 18:399-404.

Reyes, M., and Verfaillie, C.M. (2001). Characterization of multipotent adult progenitor cells, a subpopulation of mesenchymal stem cells. *Ann N Y Acad Sci* 938:231-233; discussion 233-235.

Reyes, M., Lund, T., Lenvik, T., Aguiar, D., Koodie, L., and Verfaillie, C.M. 5 (2001). Purification and *ex vivo* expansion of postnatal human marrow mesodermal progenitor cells. *Blood* 98:2615-2625.

Sauér, B. & Henderson, N. (1988). Site-specific DNA recombination in mammalian cells by the Cre recombinase of bacteriophage P1. *Proc. Natl. Acad. Sci. U.S.A* 85:5166-5170

10 Sasaki, M.S. & Tonomura, A. (1973). *Cancer Research* 33:1829-1836.

Schwartz, *et al.*, U.S. Patent No. ,759,793

Shambrott, M., Axelman, J., Wang, S., Bugg, E., Littlefield, J., Donovan, P., Blumenthal, P., Huggins, G., Gearhart, J.: (1998) Derivation of pluripotent stem cells from cultured human primordial germ cells. *Proc. Natl. Acad. Sci. U.S.A.* 15 95:13726-31.

Simmons, P., *et al.*, U.S. Patent No. 5,677,136

Strathdee, C.A. *et al.*, (1992). Evidence for at least four Fanconi anemia genes including FACC on chromosome 9. *Nature Genet.* 1:196-198

Svendsen, C. N., and Caldwell, M. A. (2000). NSCs in the developing 20 central nervous system: implications for cell therapy through transplantation. *Prog Brain Res.* 127:13-34.

Svendsen, C. N., Caldwell, M. A., Ostenfeld, T. (1999). Human neural stem cells: Isolation, expansion and transplantation. *Brain Path* 9:499-513.

Timmers, C. *et al.*, (2001). *Mol. Cell.* 7:241-248

25 Thomson, J. A., Itskovitz-Eldor, J., Shapiro, S. S., Waknitz, M. A., Swiergiel, J. J., Marshall, V. S., and Jones, J. M. (1998). ES cell lines derived from human blastocysts. *Science* 282:114-7.

Thomson, J., Kalisman J., Golos, J., Durning, M., Harris, C., Becker, R., Hearn, J. (1995) Isolation of a primate embryonic stem cell line. *Proc. Natl. Acad. 30 Sci. U.S.A.* 92:7844-8,

Tsukamoto, *et al.*, U.S. Patent No. 5,750,397

Tsukamoto, *et al.*, U.S. Patent No. 5,716,827

Tzanakakis, E.S., Hansen, L.K., and Hu, W.S. (2001). The role of actin filaments and microtubules in hepatocyte spheroid self-assembly. *Cell Motil Cytoskeleton* 48:175-189.

Watt, F. (1997). Epidermal stem cells: markers patterning and the control of stem cell fate. *Philos Trans R Soc Lond B Biol Sci* 353: 831-6.

Watt, S., Gschmeissner, S., and Bates, P. (1995). PECAM-1: its expression and function as a cell adhesion molecule on hemopoietic and endothelial cells. *Leuk Lymph* 17:229.

Williams, R. L., Hilton, D. J., Pease, S., Willson, T. A., Stewart, C. If, Gearing, D. P., Wagner, E. F., Metcalf, D., Nicola, N. A., and Gough, N. M. (1988). Myeloid leukemia inhibitory factor maintains the developmental potential of ES cells. *Nature* 336:684-7.

Whitney, M. *et al.*, (1996). A common mutation in the FACC gene causes Fanconi anaemia in Ashkenazi Jews. *Blood* 88:49-58.

Yanez & Porter. (1999). *Gene Therapy* 6:1282-1290.

Yoo, J. U., Barthel, T. S., Nishimura, K., Solchaga, L., Caplan, A. I., Goldberg, V. M., Johnstone, B. (1998). Then chondrogenic potential of human bone-marrow-derived mesenchymal progenitor cells. *J Bone Joint Surg Am*. 12:1745-57.

Young, H., *et al.*, U.S. Patent No. 5,827,735

Zambrowicz, B. P., Imamoto, A., Hering, S., Herzenberg, L. A., Kerr, W. G., and Soriano, P. (1997). Disruption of overlapping transcripts in the ROSA beta geo 26 gene trap strain leads to widespread expression of beta-galactosidase in mouse embryos and hematopoietic cells. *Proc Natl Acad Sci U S A*. 94:3789-94.

Having thus described in detail preferred embodiments of the present invention, it is to be understood that the invention defined by the appended claims is not to be limited to particular details set forth in the above description, as many apparent variations thereof are possible without departing from the spirit or scope of the present invention. Modifications and variations of the method and apparatuses described herein will be obvious to those skilled in the art, and are intended to be encompassed by the following claims.

**CLAIMS**

We claim:

1. A method of altering a first gene expression pattern in an isolated multipotent adult progenitor cell (MAPC) comprising:
  - a) introducing into a MAPC an exogenous polynucleotide molecule, wherein the exogenous polynucleotide molecule comprises i) a targeting polynucleotide sequence homologous to a genomic DNA sequence of the MAPC and ii) a donor nucleotide sequence of interest; and
  - b) culturing the MAPC under conditions sufficient to homologously recombine the exogenous polynucleotide molecule, such that a resultant MAPC has a second expression pattern different than the first gene expression pattern.
2. The method of claim 1, wherein the MAPC is isolated from a mouse, a rat or a human.
3. The method of claim 1, wherein the introducing is via nucleoporation.
4. The method of claim 1, further comprising differentiating the resultant MAPC.
5. The method of claim 1, wherein the exogenous DNA molecule further comprises a DNA sequence encoding a selectable marker.
6. A method of making recombinant multipotent adult progenitor cells (MAPCs) comprising:
  - a) culturing isolated MAPCs at low density;
  - b) nucleoporating the MAPC in the presence of an exogenous polynucleotide molecule, wherein the polynucleotide molecule comprises i) a targeting polynucleotide sequence homologous to a genomic DNA

sequence of the MAPC and ii) optionally a DNA sequence encoding a gene product; and

- c) culturing the MAPC obtained in b) under conditions sufficient to homologously recombine the exogenous DNA molecule, thereby making a recombinant MAPC.

7. The method of claim 6, wherein the MAPCs are cultured at about 500 cells/cm<sup>2</sup>.

8. The method of claim 6, wherein the MAPCs are isolated from a mouse, a rat or a human.

9. A recombinant MAPC produced by the method of claim 6.

10. A method of correcting a genetic defect in a mammal, wherein the defect is one or more defective nucleotide sequence(s) in the genome of the mammal that give(s) rise to defective gene expression, the method comprising:

- a) culturing a MAPC from the mammal having the genetic defect;
- b) introducing into the MAPC an exogenous DNA molecule, wherein the DNA molecule comprises i) a targeting DNA sequence homologous to a genomic DNA sequence of the MAPC and ii) one or more donor nucleotide sequence(s) necessary for correcting said genetic defect in said mammal;
- c) culturing the MAPC under conditions sufficient to homologously recombine the exogenous DNA molecule into the genome of the MAPC, thereby obtaining a genetically altered MAPC;
- d) selecting said genetically altered MAPC; and
- e) transplanting said genetically altered MAPC into the mammal, wherein the selecting and transplanting can be done in any order or simultaneously.

11. The method of claim 10, wherein the mammal is a mouse, a rat or a human.

12. The method of claim 10, wherein the genetic defect is in the gene encoding FANCC.
13. The method of claim 10, wherein the introducing is via nucleoporation.
14. The method of claim 12, wherein the selecting is by treatment of the MAPCs with a dose of mitomycin C, wherein the dose is toxic to MAPCs not expressing the gene product and non-toxic to said genetically altered MAPC expressing the gene product.
15. The method of claim 10, further comprising differentiating the genetically altered MAPC prior to or upon transplanting.
16. The method of claim 10, wherein the exogenous DNA molecule further comprises a DNA sequence encoding a selectable marker.
17. The method of claim 16, wherein the DNA sequence encoding the selectable marker is flanked at each of the 5' and 3' ends by a *lox P* site.
18. A genetically altered MAPC comprising an exogenous polynucleotide molecule homologously recombined into the genome of a MAPC.
19. A differentiated cell arising from the genetically altered MAPC of claim 18.
20. A method of expressing a functional gene product of interest in an isolated MAPC having a defective nucleotide sequence from which a functional gene product cannot be expressed, the method comprising:
  - a) introducing into the MAPC an exogenous DNA molecule, wherein the DNA molecule comprises i) a targeting DNA sequence

homologous to a genomic DNA sequence of the MAPC and ii) a donor nucleotide sequence corresponding to the defective nucleotide sequence; and

- b) culturing the MAPC under conditions sufficient to homologously recombine the exogenous DNA molecule into the genome of the MAPC, thereby expressing the functional gene product of interest in said MAPC.

BEST AVAILABLE COPY

Figure 1

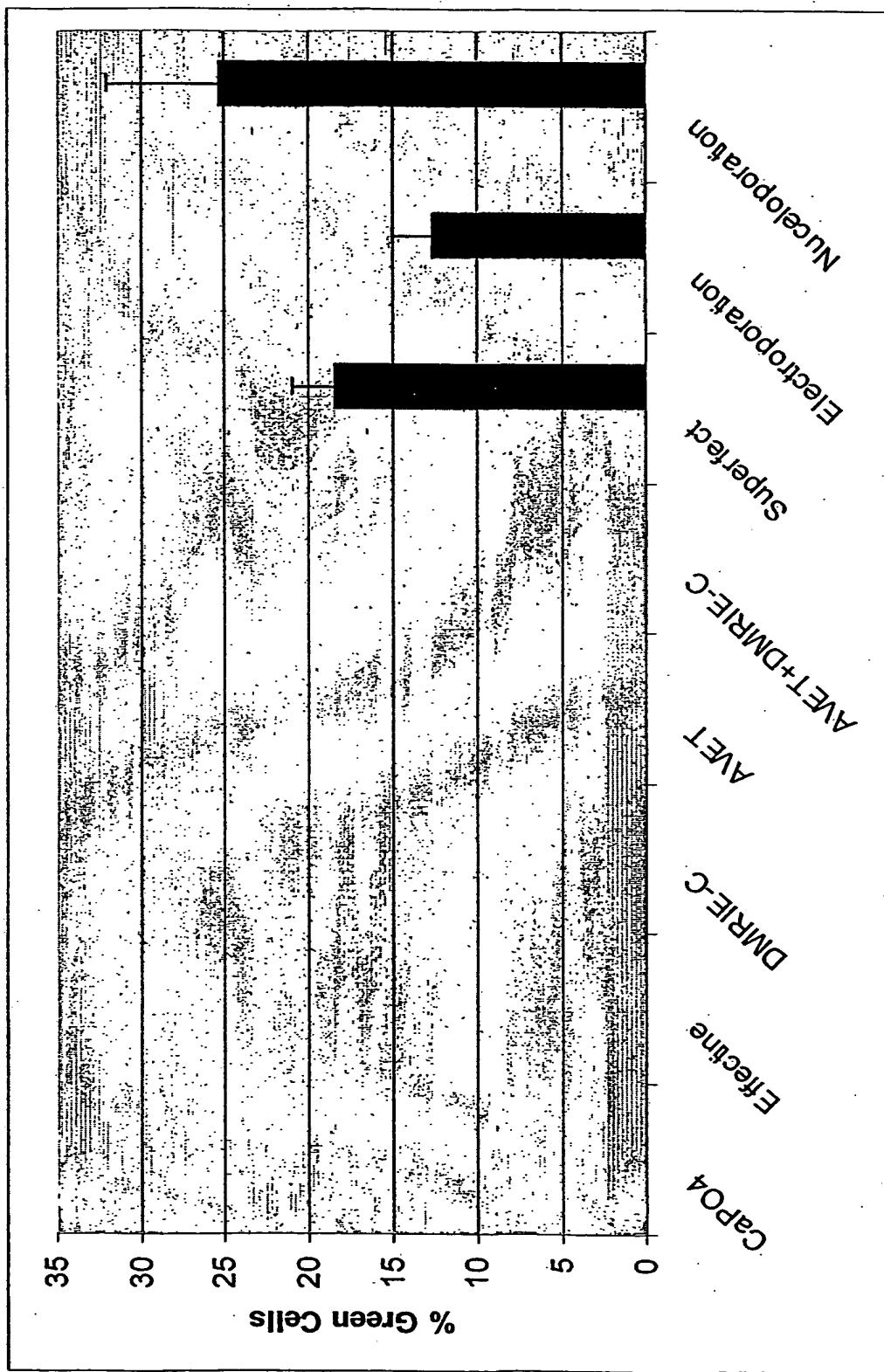
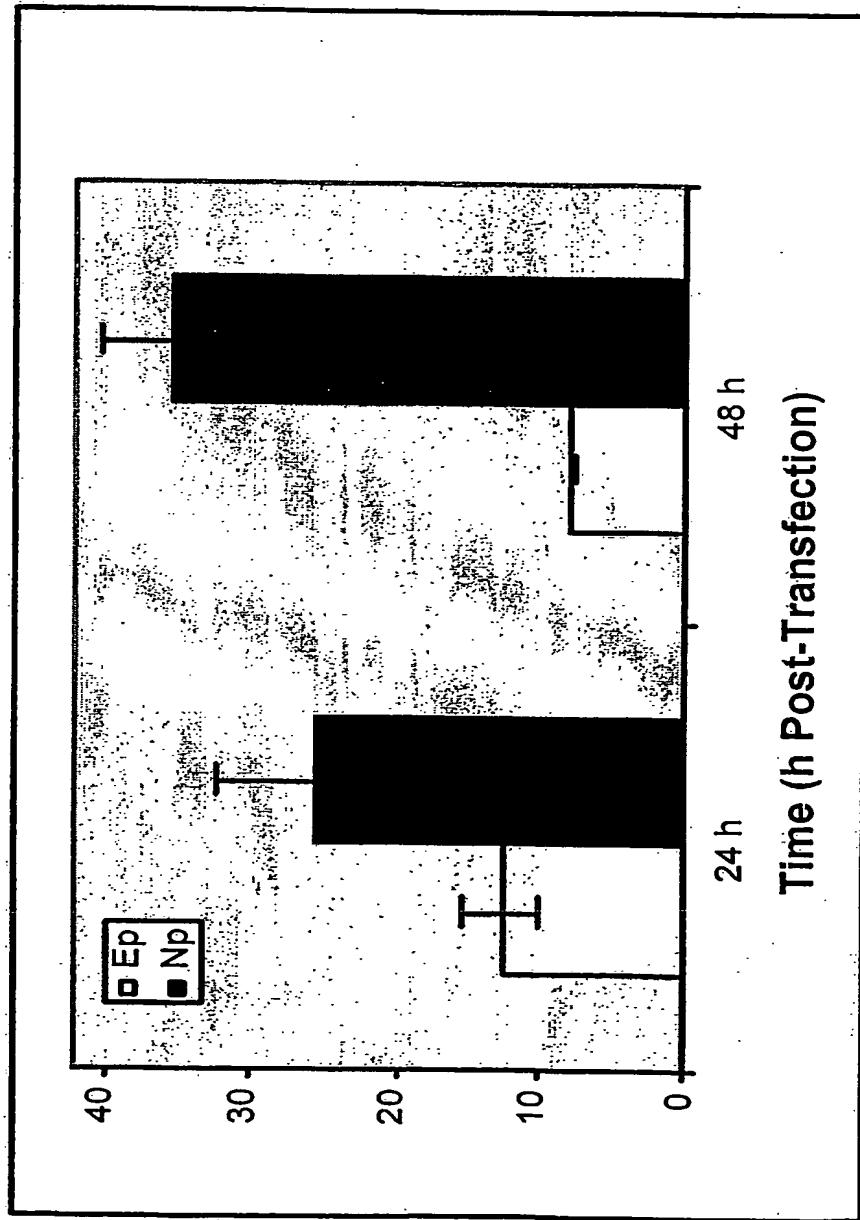


Figure 2



BEST AVAILABLE COPY

Figure 3

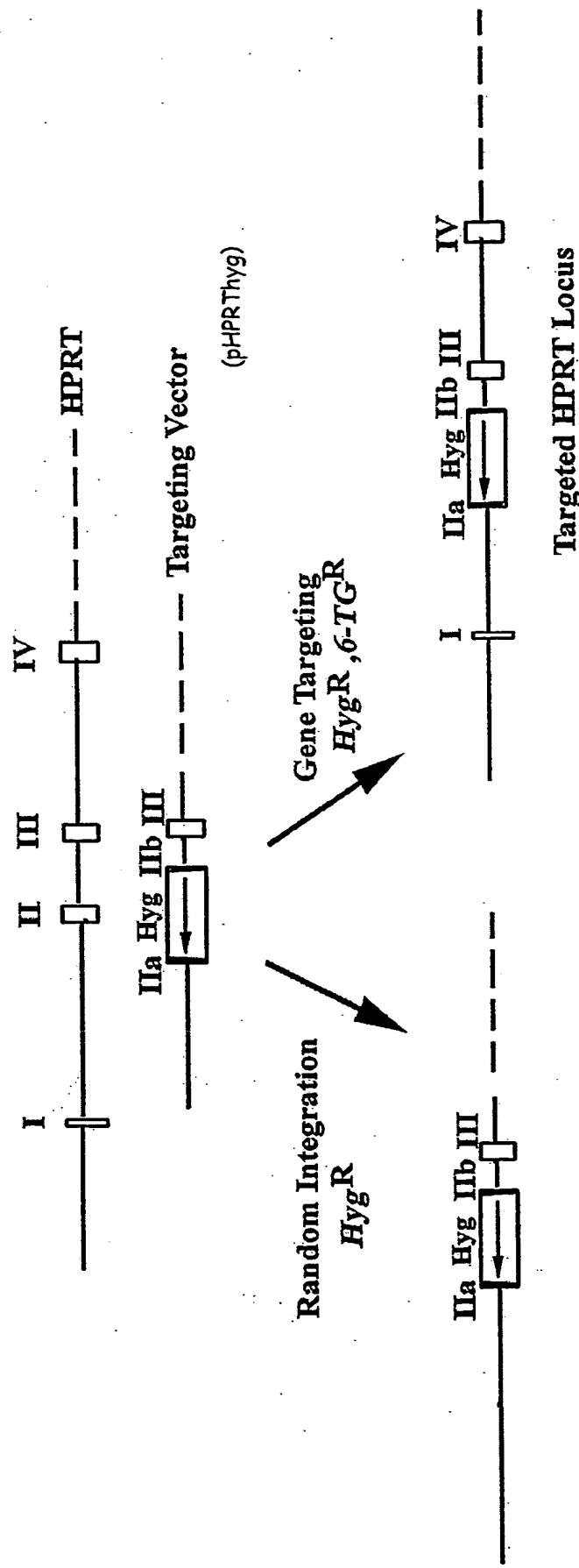


Figure 3

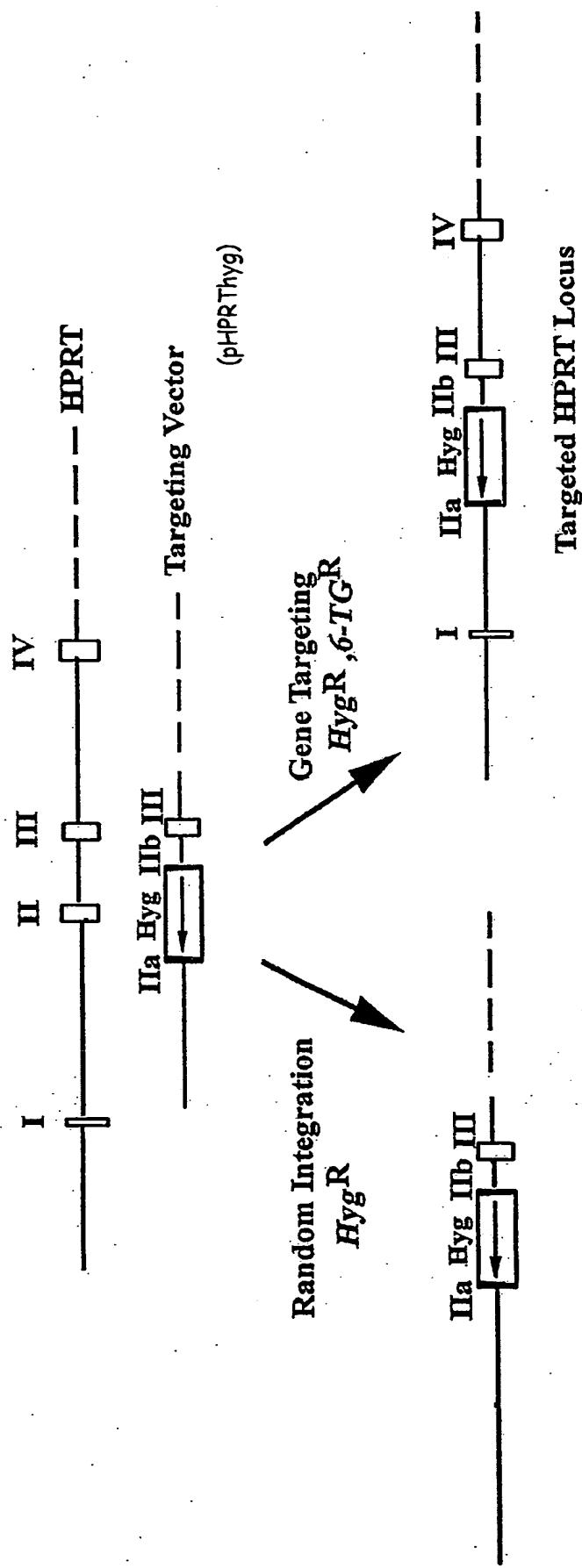
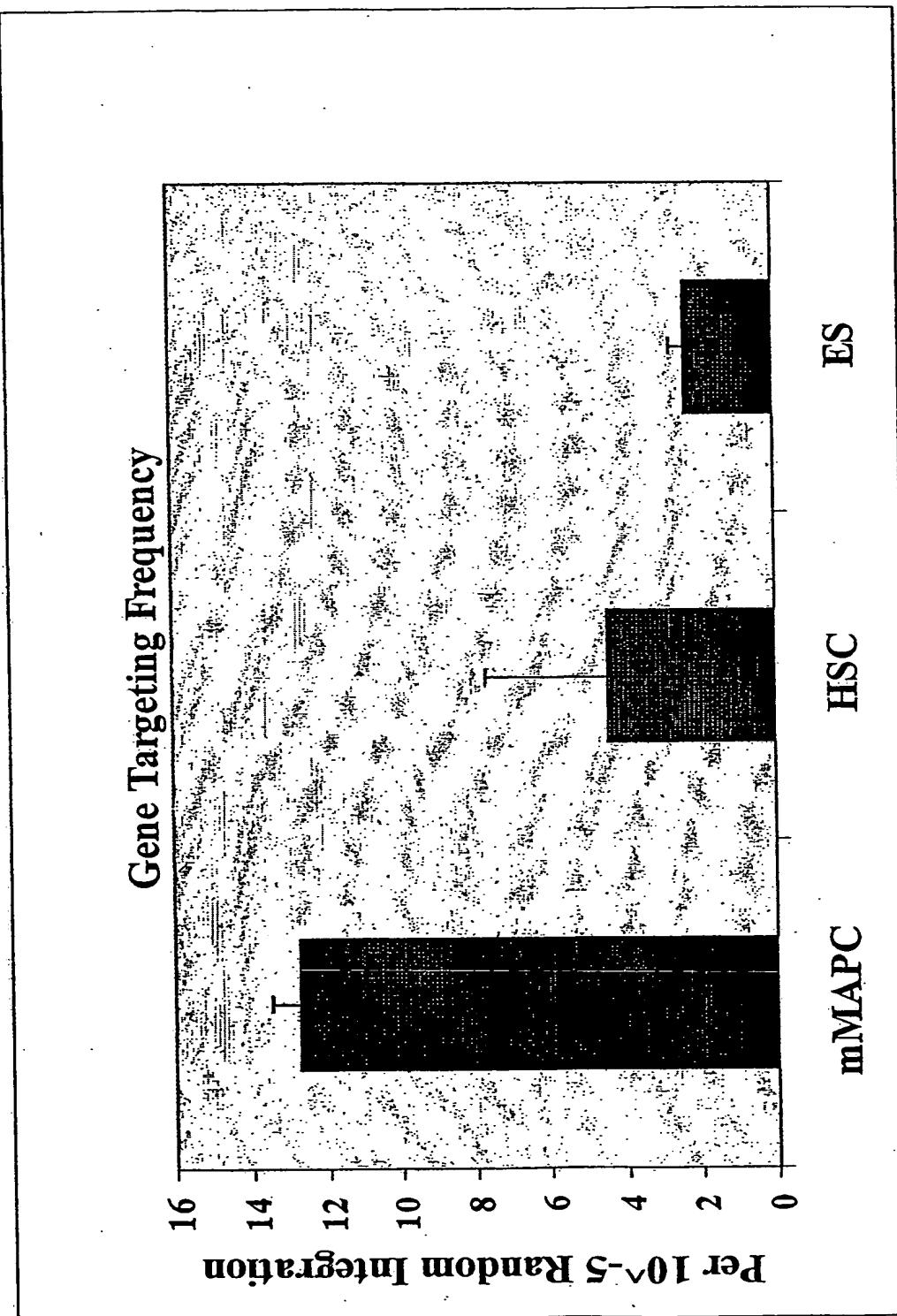


Figure 4



BEST AVAILABLE COPY

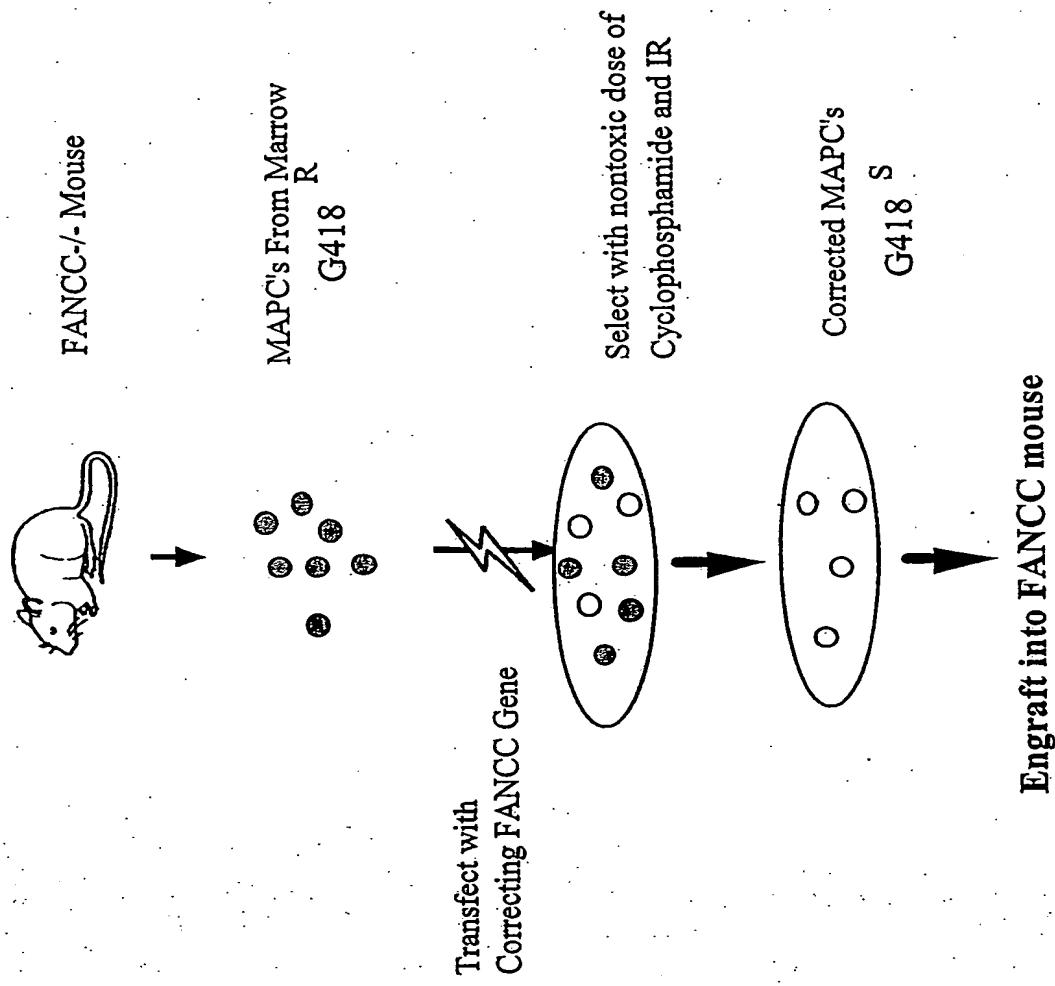
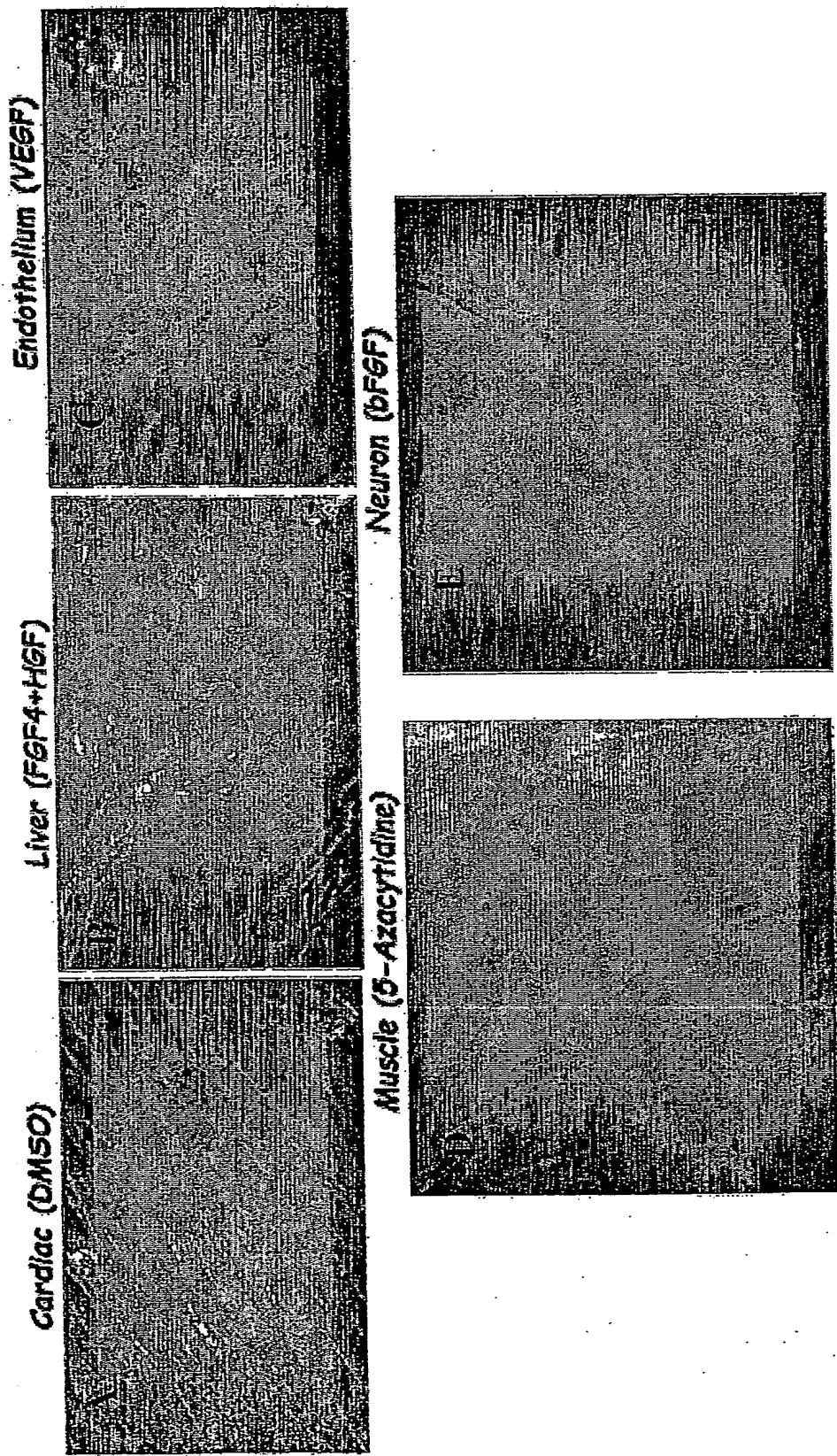
**Figure 5**

Figure 6



Differentiation-Day 11

BEST AVAILABLE COPY

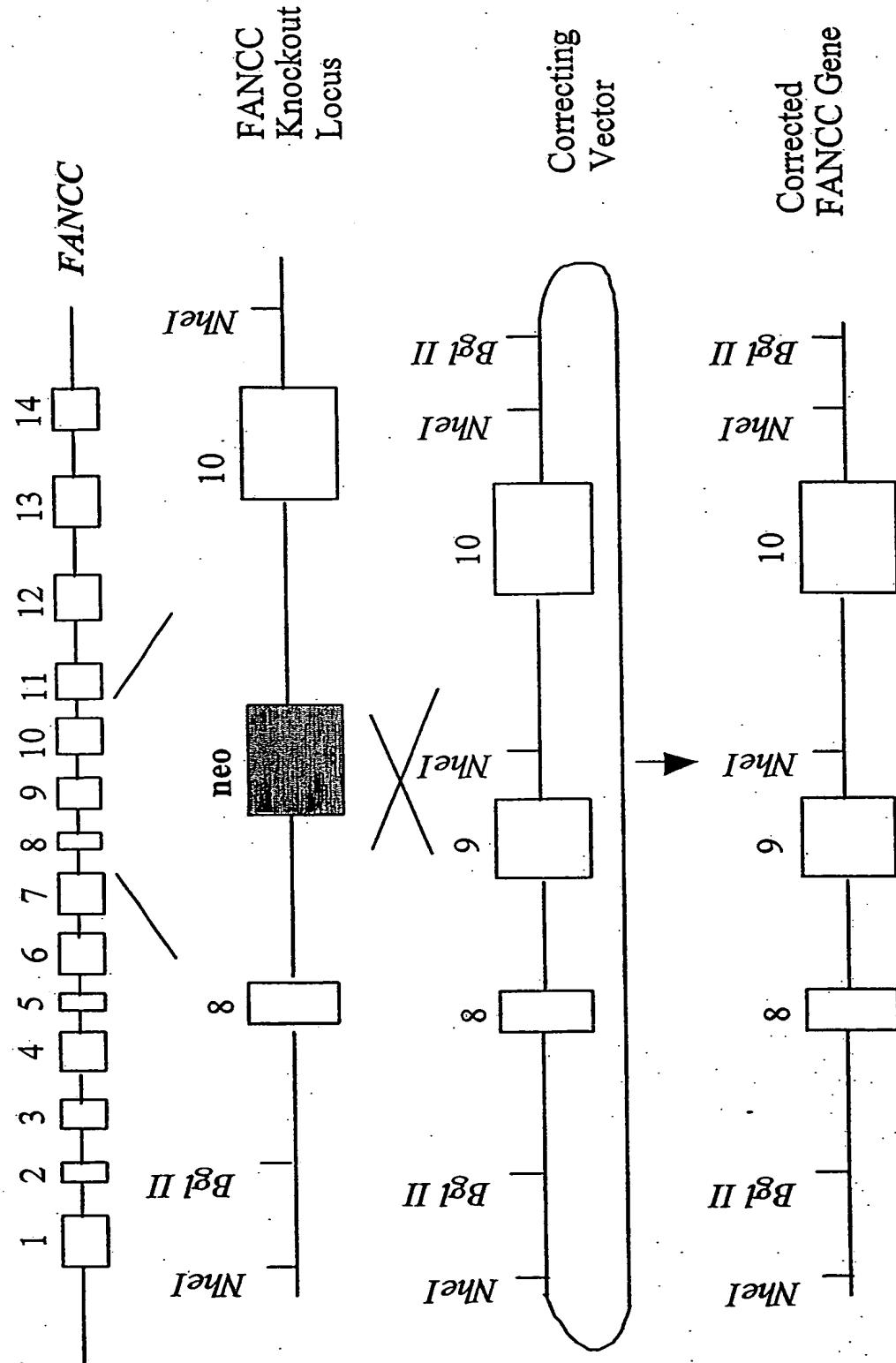
**Figure 7**

Figure 8

ATGGCTCAAGATTCAGTCACTTCTGTGATTATCAGTTGGATGCA 50  
 GAAGCTTCTGATGGATCAGGCTCCACTTGGAAACCCAGCAAGACA 100  
 CCTGCTTCACTGGCTCAGTCCAGGGAGTTCTTAAGGAAGATGTGAA 150>  
 GCCTGAAAGAGATGGATTCTAATACAGTCATGAAAGATTCCACAAT 200>  
 TGGTCAACTGTTGGCAAAAGCTTGTGGAATCTCTTATTTAGCATATG 250>  
 ATGAAAGCCAATAATTCTAATATGGCTTATGTTGTTAAATTACAAA 300>  
 GAACCAACAGAATTCGGACAAATCAAACCTAAACTTAACTCCTGGATAACGGGTGT 350>  
 ATTATCTCATATACATTCAAGCACTCAGATTGATAAAGAAGTTGCTCTT 400>  
 TCACTCAAGGTCTTGGGATGGCACCTATAGATTAATCTGGTGCCT 450>  
 AAAAATATGGTTTATCATTAGCGTCTGAACAGATGTTCACTTTAA 500>  
 TGGATTAAACACTCAAGCGGAATGGCTCCCGAGCGAGTGGCGTCCCTGGTG 550>  
 CACGAGTTTGTGTCCTCACCTTACCTGACAGATGTTGACCCCCCTGGTG 600>  
 GAGGCTCTCCTCATCTGTATGGACGTGAACCTCAGGAATTCAGCTCAGCC 650>  
 AGAGTCTTGTGAGGGCTGTAACCGAGGCCATTGTGTAAGAAAGATTTCTC 700>  
 TCCCCATGTCAGCTGTAGTCTGCCCCCTGGCTCTGGCACCTTCCAGCCTT 750>  
 GAAAAAGCAATGCTGCATCTTGTAAAGCTAATCTCAGTGGAGAGAAA 800>  
 TTGTCTGAGAAGGATCGAATGCTTTATAAAAGATTCACTGGCTGCCTCAAG 850>  
 CAGCCGCCACCCCTGCCATATTCCGGGTTGTGATGAGATGTTAGGTGT 900>  
 GCACCTCTGAAACCGATGGCCCTGGAAATCATAGCCACTATTAGGT 1000>  
 GTTTACGGCAGTGTCTTGTAGAAGCTCTGGAGAAAGCAAGCAGCTGC 1050>  
 GGTGTTGCACTCAAGACCTACTTCCCTTACACTTCTGCCATG 1100>  
 GTGCTGCTGCAAGACCTCAAGATACTCCCTGGGACACTGGCTCAGAC 1150>  
 ACTGAAGCAATTCTGAACTGCTCAAGAAGCAAGCTGGAAAGCAACTC 1200>  
 ATGGGTCTCTGGGAGGTCTCCCTTGTAGAGCTGTTCTGTTCAATTCACTTC 1250>  
 GGAGGATGGGCTGAGATGGTGGCAGAGCAATTACTGATGTCAGGACTCGCA 1300>  
 ACCCCCCACGGCCCTGGCTCTGGCTCTACTACGGCCCCGGTGGGGC 1350>  
 ATGGGAGGGCAGAGGCAAGACAGACTATGGTCCAGGTGAAGGGCGTGGGGC 1400>  
 CACCTCTGGCAATGTCCAGAAGCAGGCACTTCAGGACTCTCAGGACTCGCA 1450>  
 GACGGTAGCAGGACAGGGCACAGACAGACCTCAGAGCTCAGGCTCTGGGGC 1500>  
 AGCTGATCAGGCACCTTCTCCTCAACTTCCTGCTCTGGCTCTGGGGC 1550>  
 CACACGATCGCTGGGATGTCATCACCCCTGATGGCTCACACTGCTGAGAT 1600>  
 AACTCACGAGATCAATTGGCTTCTTGTACAGATGGAAATC 1650>  
 GTCTTGGCATTGAAAGGCCCTAGATCAGAAGAAACTGGCCGAGAGCTCCTT 1700>  
 AAAGGAGCTGGAACTCAAGGTCTAG 1724

Figure 9

MAQDSVDLSCDDYQFWIMQKLSWWDQASTLETTQQDTCLHVAQFQEEFLRKMYE 50  
ALKEMDSNTVIERFPTIGQLLAKACWNPNFTILAYDESQKILIIWCLCCLINK 100  
EPQNSGQSKLNNSWIQGVLSHILSALIREDFKEVALFTQGLGYAPIDYYPGLL 150  
KNMVLSSLASELRENHLNGENTQRRAPEVASSLSRVCVPLITLTDVDPLV 200  
EALLLICHGREPQEIIQPEFFFEAVNEAIIKKISLPPMSAAVVCVLIWHLPLSL 250  
EKAMLHLLFEKLISSERNCLRRIEFCIFDSSLQAAACHPAIFRVRVDEMFRC 300  
ALLETDGALEIATIQVFTQCFVEALEKASKQLRFALKTYFPYTSPSLAM 350  
VLLQDPQDIPRGHWLQTLKHISELLIREAVIDQTHGSCGGPFESWFLFIHF 400  
GGWAEMVAEQILLMSAAEPPTALLWLLAFLYYGPRDGRQRAQTMVQVKAVLG 450  
HLLAMSRSSSISQAQDLQTVAGQGTDTDLRAPAPAQQQLIRHILLNELLWAPGG 500  
HTIIAWDVITLMAHTAEITHELIGFLDQTLYRWNRLGIESPRSEKLLARELL 550  
KELRTQV. 558

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US03/38811

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C12N 15/85; A61K 48/00  
US CL : 435/325; 514/44

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/325; 514/44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
STN: EMBASE BIOSIS CAPLUS

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages   | Relevant to claim No. |
|------------|--|-----------------------|
| A          | Database EMBASE on STN, LAKSHMIPATHY, U et al. 'A Non-viral Method for Efficient Transfection of Embryonic and Adult Stem Cells.' Blood, 16 November 2003, Vol. 102, No. 11, pp. 492b, see Abstract. | 1-20                  |

Further documents are listed in the continuation of Box C.

See patent family annex.

- Special categories of cited documents:
- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier application or patent published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- "&" document member of the same patent family

Date of the actual completion of the international search

09 May 2004 (09.05.2004)

Date of mailing of the international search report

28 MAY 2004

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US  
Commissioner for Patents  
P.O. Box 1450  
Alexandria, Virginia 22313-1450

Facsimile No. (703) 872-9306

Authorized officer

Celine Qian, Ph.D.

Telephone No. (571) 272-1600